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EXAMINATION OF INDIAN SHARK-LIVER OILS FOR
VITAMIN A AND SOME ANALYTICAL
CHARACTERISTICS.

RELATIONSHIP BETWEEN THE EXTINCTION COEFFICIENT OF
CARR-PRICE REACTION AT ITS ABSORPTION MAXIMUM
AND INTERNATIONAL UNITS.

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INFORMATION regarding the physical and chemical properties of Indian shark-liver oils is meagre and the limits of variations of the different analytical values are not available. In this paper, the analytical values which have a more or less direct bearing on the keeping quality of shark-liver oils have been reported.

EXPERIMENTAL.

Twenty-six genuine specimens of commercial shark-liver oils were obtained from Madras, Travancore and Bombay and were stored in the refrigerator up to the time of experiments. Samples Nos. 27 and 28 were diluted with refined ground-nut oil by the suppliers.

Standard methods were followed for the determination of acid, saponification and acetyl values. The first few samples were tested for the iodine values both

by the standard Wij's method as well as by the Rosenmund and Kuhnheim's method as described by Vere-Zones (1936). The other samples were analysed by the latter method only, as it was found to be more convenient. The figures obtained by the Wij's method were found to be slightly higher than those by the other method.

Peroxide number was determined by the Wheeler's (1932) method as modified by Stansby (1941) and was expressed in terms of millilitres of 1.0 N thiosulphate per kilogram of oil. The unsaponifiable matter was determined with a slight modification of the method recommended by the Society of Public Analysts (1933). The iodine value of the unsaponifiable matter was determined by the Rosenmund and Kuhnheim's method and the iodine value of the glycerides was calculated from the iodine values of the whole oil and of the unsaponifiable fraction.

The moisture content of the oil was estimated from the loss in weight on drying the oil at the temperature of boiling water, taking the necessary precautions for obviating chances of oxidation of the oil and consequent gain in weight. The oil was spread in a thin layer in a conical flask closed with a rubber-cork, having two glass-tubes one of which terminated just above the oil-layer and the other below the cork. Through the first tube nitrogen dried by bubbling through concentrated sulphuric acid was passed in a slow but steady stream. A long calcium chloride tube was attached to the end of the other tube so as to facilitate quick removal of moisture from the oil and to prevent contamination with any atmospheric moisture during drying.

The induction period of the oil was determined at 65°C. by the oxygen-absorption method (Bose and Banerjee, 1945).

Vitamin A was estimated by three different methods: (i) Lovibond tintometric method as developed by Carr and Price (1926) and modified by Coward *et al.* (1931); (ii) Pulfrich photometric method (using the filter S.61) as standardized in this laboratory (Rao, 1944); (iii) Hilger vitameter A method as recommended by the Vitamin Assay Committee of the American Drug Manufacturers' Association (1937). The Hilger vitameter A which was previously standardized against a Hilger quartz spectrophotometer in this laboratory (Rao, *loc. cit.*) was used for this study. The results obtained by (i), (ii) and (iii) were expressed as Carr-Price blue values, $B_{1\text{ cm.}}^1\%$ and $E_{1\text{ cm.}}^1\%$ 328m μ values respectively. For converting $E_{1\text{ cm.}}^1\%$ 328m μ values into international units per gramme, the factor 1,600 as recommended for fish-liver oils by the International Conference on Vitamin Standardization, 1934 (quoted by Edisbury, 1940) was used. For the isolation of the unsaponifiable fraction required for vitamin A estimation, the method of Morgan *et al.* (1935) was followed with a slight modification.

The results of the analyses are given in Tables I, II and III. The iodine and saponification values are given to the nearest integer.

TABLE I.
Analytical characteristics of shark-liver oils.

Sample of oil.	IODINE VALUE (whole oil).		Sapon. value.	Acetyl value (apparent).	Peroxide number.	Induction period at 65°C.	Moisture, per cent.	Unsatapon. matter, per cent.	Iodine value of unsapon. matter.	Iodine value of glycerides (calculated).
	Wij's method.	Rosenmund and Kuhnheim's method.								
A	0.54	145	144	14.6	0	6.5	1.05	7.18	170	142
G	1.86	158	154	17.8	1.0	4.5	0.88	11.18	186	150
3	5.88	145	140	16.0	4.2	6.2	1.20	5.55	132	141
4	2.00	146	145	18.2	3.1	6.2	1.22	6.82	158	144
5	0.52	160	158	13.9	0	5.5	0.26	11.09	190	154
E	1.32	145	142	14.9	1.2	8.5	1.58	7.52	142	142
7	0.46	152	150	15.2	0	8.5	1.09	7.90	173	148
C	0.81	152	146	13.0	0	8.5	2.00	7.39	184	143
9	3.85	148	144	16.9	0	4.5	0.85	1.55	142	144
10	7.42	158	155	19.9	4.6	4.5	0.88	2.45	155	154
H	19.37	155	154	38.2	6.0	3.5	0.55	8.42	176	152
D	2.65	...	152	16.5	0	7.4	0.95	2.22	155	152
K	0.08	...	156	12.8	0.8	7.4	0.88	10.52	190	152

Examination of Indian Shark-Liver Oils for Vitamin A.

TABLE I—*concl'd.*

Sample of oil.	Acid value.	IODINE VALUE (whole oil).		Sapon. value.	Acetyl value (apparent).	Peroxide number.	Induction period at 65°C.	Moisture, per cent.	Unapon. matter, per cent.	Iodine value of unapon. matter.	Iodine value of glycerides (calculated).
		Wij's method.	Rosenmund and Kuhnheim's method.								
14	10.25	...	150	152	24.3	5.5	4.2	0.80	8.08	184	147
15	6.33	...	156	190	18.6	6.0	4.0	1.10	1.92	156	156
16	6.02	...	163	156	20.3	3.8	5.2	0.99	13.27	191	159
17	12.84	...	155	155	28.1	3.8	4.5	0.88	10.47	189	151
18	11.21	...	141	184	19.3	2.5	4.5	1.09	2.34	141	141
F	0.98	...	144	160	14.1	0	8.8	1.08	6.99	143	144
I	14.36	...	147	162	30.3	6.2	3.8	1.01	6.81	161	146
B	5.18	...	149	150	18.6	0.4	5.0	2.04	9.48	188	145
22	15.75	...	162	160	26.9	0.8	4.0	0.95	8.63	188	161
23	11.89	...	158	158	21.5	1.8	4.0	0.92	8.75	192	155
24	23.05	...	145	140	33.7	5.8	4.0	1.00	12.07	132	147
25	0.74	...	150	170	12.1	0.5	3.5	1.28	4.88	189	148
26	3.09	...	155	156	17.3	1.0	5.5	2.09	9.92	183	152
27	0.55	...	122	194	9.9	0	8.8	0.51	1.99	131	122
28	0.78	...	122	192	10.8	0	8.8	0.82	1.58	140	122

TABLE II.

Vitamin A values of shark-liver oils : relationship between $B_1\%$ cm. values and international units.

Sample of oil.	Vitamin A Carr-Price blue values (unsap. frac.).	VITAMIN A $B_1\%$ cm.		$\frac{b}{a}$	VITAMIN A $E_1\%$ cm.		$\frac{c}{d}$	$\frac{b}{d}$	Vitamin A per gramme (calculated) $d \times 1,600$	$\frac{I. U.}{a}$	$\frac{I. U.}{b}$
		Whole oil. a	Unsap. frac. b		Whole oil. c	Unsap. frac. d					
A	130.1	6.83	9.30	1.36	4.22	3.58	1.18	2.59	5,728	838.6	615.9
G	58.3	2.53	3.74	1.48	1.87	1.40	1.34	2.66	2,240	885.3	598.8
3	286.8	22.22	23.11	1.04	13.19	12.56	1.05	1.84	20,096	904.4	869.8
4	191.6	11.36	13.52	1.19	7.76	7.19	1.08	1.88	11,504	1,012.6	650.8
5	131.9	6.49	8.18	1.26	3.86	3.36	1.15	2.43	5,376	828.5	657.2
E	115.0	5.40	7.02	1.30	3.13	2.80	1.12	2.50	4,480	829.6	638.3
7	323.8	26.12	26.65	1.02	14.29	13.74	1.04	1.94	21,984	841.6	824.9
C	180.7	8.25	10.06	1.22	5.64	4.74	1.19	2.12	7,584	919.1	753.9
9	95.2	3.74	6.03	1.61	3.18	2.38	1.34	2.53	3,808	1,018.1	631.6
10	185.4	6.08	7.49	1.23	6.75	6.14	1.10	1.22	9,824	1,615.7	1,311.6
H	60.3	3.96	5.50	1.39	2.67	2.09	1.28	2.62	3,344	844.3	607.8
D	100.5	7.32	8.93	1.22	4.54	3.55	1.28	2.51	5,680	775.9	635.9
K	114.5	8.02	11.48	1.43	5.59	4.30	1.30	2.67	6,880	857.8	599.2
14	183.6	15.20	16.42	1.08	9.91	8.25	1.08	1.99	13,200	868.4	803.8

TABLE II—*concl'd.*

Sample of oil.	Vitamin A Carr-Price blue values (unsep. frac.).	VITAMIN A B 1 %		$\frac{b}{a}$	VITAMIN A E 1 %		$\frac{c}{d}$	$\frac{b}{d}$	Vitamin A I. U. per gramme (calculated) $d \times 1,600$	$\frac{\text{I. U.}}{a}$	$\frac{\text{I. U.}}{b}$
		Whole oil. a	Unsep. frac. b		Whole oil. c	Unsep. frac. d					
15	66.5	3.32	5.16	1.55	2.40	2.00	1.20	2.58	3,200	963.8	620.1
16	276.8	20.95	23.05	1.10	11.75	11.41	1.03	2.02	18,256	871.4	792.0
17	136.5	9.59	12.28	1.28	5.84	4.95	1.18	2.48	7,920	825.8	644.9
18	159.8	13.16	14.35	1.09	6.59	6.28	1.05	2.19	10,048	763.5	700.2
F	188.2	10.92	12.99	1.19	7.44	6.83	1.09	1.90	10,928	1,000.7	841.2
I	74.2	3.63	4.93	1.36	2.44	1.85	1.32	2.66	2,960	815.5	600.5
B	99.9	6.86	8.78	1.28	4.58	3.67	1.25	2.39	5,872	855.9	603.6
22	103.0	4.48	7.45	1.66	3.63	2.98	1.22	2.50	4,768	1,064.2	639.8
23	57.1	2.47	4.21	1.70	2.07	1.62	1.28	2.59	2,592	1,049.3	615.6
24	240.6	11.91	17.87	1.50	9.58	9.31	1.03	1.92	14,896	1,250.7	833.5
25	203.6	10.44	13.89	1.33	7.96	7.51	1.06	1.85	12,016	1,150.9	865.0
26	95.2	5.97	8.42	1.41	3.57	3.19	1.12	2.64	5,140	861.0	610.5
27	11.7	0.72	1.22	1.69	0.52	0.41	1.28	2.97	656	911.1	537.7
28	31.3	1.44	2.49	1.72	1.17	0.93	1.26	2.68	1,488	1,033.3	597.5
	Average for samples		...	1.34	1.17	2.31	...	944.8	713.0

TABLE III.

Use of the conversion factors for expressing $B \frac{1}{1} \%$ values into international units.

Sample of oil.	VITAMIN A EXPRESSED AS :						PERCENTAGE DIFFERENCE*.	
	$B \frac{1}{1} \%$		$E \frac{1}{1} \%$ (unsap. matter). <i>d</i>	I. U. per g. (calculated).			Whole oil.	Unsap. matter.
	Whole oil. <i>a</i>	Unsap. matter. <i>b</i>		<i>d</i> × 1,600	<i>a</i> × 945	<i>b</i> × 713		
29	7.33	11.73	4.51	7,216	6,926	8,364	-4.02	+15.90
30	3.81	6.40	2.68	4,288	3,599	4,563	-16.07	+6.41
31	17.68	19.80	8.32	13,312	16,708	14,117	+25.51	+6.04
32	9.61	14.90	6.01	9,616	9,080	10,623	-5.57	+10.47
33	7.16	11.32	4.42	7,072	6,766	8,072	-4.32	+14.14
34	8.73	13.02	5.21	8,336	8,249	9,281	-1.04	+11.33
35	22.75	24.57	12.22	19,552	21,499	17,518	+9.95	-10.40

* These figures represent the per cent that the international units calculated from the $B \frac{1}{1} \%$ values are more or less than the international units calculated from $E \frac{1}{1} \%$ values.

DISCUSSION.

The acid values for most of the samples examined were undesirably high. Most of the oils of higher acidity had usually dark colour compared to the light-yellow colour of the oils of lower acidity and had an unpleasant sour odour.

The variations in the characteristics were maximum with the samples Nos. 27 and 28, because these were diluted with refined ground-nut oil by the suppliers so as to bring down the potency to a standard level. For the remaining 26 samples examined, the saponification values varied from 140 to 190, the acid values from 0.46 to 26.05, the acetyl values from 12.1 to 38.2, the iodine values from 140 to 163, the unsaponifiable matter from 1.55 to 13.27 per cent, the iodine values of the unsaponifiable matter from 132 to 192, peroxide number from 0 to 6.2, induction period at 65°C. from 3.5 to 8.8 hours, moisture content from 0.26 to 2.09, and the iodine values (calculated) of the glycerides from 141 to 161. Evers *et al.* (1936) obtained fairly constant iodine values for the glycerides of different samples of halibut-liver oils.

The acetyl value appeared to increase more or less with the increasing free fatty-acids content of the oils, the acetyl values of the oils having higher acid values having been found appreciably higher than those of the oils having lower acid values.

As may be normally expected, the saponification value decreased with increasing unsaponifiable matter content of the oils. In the first few samples, there was some correlation between the unsaponifiable matter content and the iodine value, but, later, no consistent relation could be established. It was obviously a mere coincidence that the oil (sample No. 16) containing the highest amount of unsaponifiable matter was found to have the highest iodine value obtained for any of the samples analysed.

Furthermore, there was no relationship between the iodine value and the induction period of the oil. This is to be expected, because the induction period of an oil depends not only on its unsaturation but also on the relative proportion of the presence of natural anti-oxidants and pro-oxidants. However, with oils of higher iodine values, the rate of absorption of oxygen after the induction period, was usually found to be faster.

The vitamin A potency for the 35 samples analysed varied from 656 to 21,984 I. U. per gramme of oil.

The $B_{1\text{ cm.}}^{1\%}$ values for the unsaponifiable fractions were found to be invariably higher than those for the whole oils. The ratio, $B_{1\text{ cm.}}^{1\%}$ value (unsap. matter) : $B_{1\text{ cm.}}^{1\%}$ value (whole oil), ranged between 1.02 and 1.72 for the 28 samples analysed, the average being 1.34. The ratio tended to be lower for the richer oils and higher for the poorer oils. This suggested the presence of some saponifiable substance in the oil, which inhibited the development of maximum blue colour. Rajagopal (1941) showed that the Carr-Price blue values for the unsaponifiable fractions of shark-liver oils were higher than those for the whole oils by 1.60 times on the average.

On the other hand, the $E_{1\text{ cm.}}^{1\%}$ $328m\mu$ values were found to be lower for the unsaponifiable portions than for the whole oils. The ratio, $E_{1\text{ cm.}}^{1\%}$ (whole oil) : $E_{1\text{ cm.}}^{1\%}$ (unsap. matter), was found to range between 1.03 and 1.34, the average being 1.17. This ratio also tended to be lower for the high potency oils than for the low potency oils. The higher extinction coefficients for the whole oils were due to some factors other than vitamin A, which could have appreciable absorption at $328m\mu$ and could be removed by saponification. Edisbury (*loc. cit.*) demonstrated that the higher values of the margarines, when tested directly, were due to the considerable irrelevant absorption by the glycerides at $328m\mu$.

The ratio, $B_{1\text{ cm.}}^{1\%}$: $E_{1\text{ cm.}}^{1\%}$, for the unsaponifiable portion of the oil, was found to vary from 1.84 to 2.68 (neglecting only one lowest value 1.22 and one highest value 2.97), the average being 2.31 for 28 samples. The ratio reported by Rao (*loc. cit.*) for whole oils was 1.92, the average for 10 samples of shark-liver oils.

While the factor 1,600 was internationally accepted for converting $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ into I. U., there is no similar conversion factor for the $B_{1\text{ cm.}}^{1\%}$ values.

Rao (*loc. cit.*) reported that the factor for converting $B_{1\text{ cm.}}^{1\%}$ values for whole oils into I. U. varied with a wide range of 500 to 1,430 in 10 samples of shark-liver oils. In this investigation also, it was observed that when whole oil was used, the conversion factor varied enormously ranging from 763 to 1,616, average being 945 for 28 samples. The range of variation was, however, narrower when the unsaponifiable fractions were used and was found to be between 597 and 870 (neglecting only two figures, one, the lowest value 538 and the other, the highest value 1,312), the average being 713.

In the case of the other seven samples of oils (Table III), no additional advantage of using the conversion factor for unsaponifiable fraction only was found. However, the use of the factor for the whole oils was not very satisfactory as was obvious from the wide range of variation in the case of the first 28 samples. Vitamin A potency in terms of I. U. is well known to the manufacturers and to the public. If for this reason it is necessary to express $B_{1\text{ cm.}}^{1\%}$ values into I. U., only approximate results can be obtained by using the conversion factor 713, the values being determined on the unsaponifiable fractions.

SUMMARY.

1. Twenty-eight samples of shark-liver oils were analysed for various analytical characteristics.

2. Thirty-five samples were examined for vitamin A potency on the whole oils as well as on the unsaponifiable fractions by (i) Lovibond tintometer, (ii) Pulfrich photometer and (iii) Hilger vitameter A.

3. Comparison was made between the vitamin A values determined on the Pulfrich photometer and Hilger vitameter A both for whole oils and the unsaponifiable fractions.

The ratio $\frac{B_{1\text{ cm.}}^{1\%} \text{ S.61}}{E_{1\text{ cm.}}^{1\%} 328m\mu}$ for unsaponifiable fractions was found to be 2.31 on the average for 28 samples.

The ratio $\frac{\text{I. U. per g.}}{B_{1\text{ cm.}}^{1\%} \text{ S.61}}$ was on the average 945 for whole oils and 713 for unsaponifiable fractions. There was less variation in the ratios for unsaponifiable fractions than for the whole oils.

4. The $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ values were found to be lower for the unsaponifiable fractions than for the whole oils.

The ratio $\frac{E_{1\text{ cm.}}^{1\%} \text{ (whole oil)}}{E_{1\text{ cm.}}^{1\%} \text{ (unsap. matter)}}$ was on the average 1.17.

5. The $B \frac{1}{1 \text{ cm.}}$ S.61 values for the unsaponifiable fractions were found to be higher than those for the whole oils. The average ratio of $B \frac{1}{1 \text{ cm.}}$ value determined via unsaponifiable matter to that obtained directly was 1.34.

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EFFECT OF SOME FACTORS ON THE PROTECTION OF VITAMIN A IN SHARK-LIVER OIL BY ANTI-OXIDANTS.

BY

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THE influence of acidity and moisture and of diffused and direct sunlight on the protective action of anti-oxidants has been studied with reference to the combination of iso-butyl gallate and citric acid used in shark-liver oils. This combination, having been found the most effective among all the anti-oxidants tested (Bose and Banerjee, 1945), has been selected for this study.

Literature on the effect of free fatty acids on the protective power of anti-oxidants is lacking. Data on the influence of moisture on anti-oxygenic action are meagre. Newton (1932) found that moisture destroyed the activity of anti-oxidants. Greenbank and Holm (1934) also noted that in presence of traces of water the acid-type anti-oxidants were not so effective as they were in dry oils. Holmes *et al.* (1936), however, reported that the period for the complete destruction of vitamin A in halibut-liver oil protected with hydroquinone and lecithin at various levels was increased by about an hour in the presence of about 7 per cent water.

Since commercial samples of pure shark-liver oil were found to have acid values ranging from 0.5 to 20.0 (occasional samples having as high as 26.0) and percentage of moisture varying from 0.26 to as high as 2.09, as reported in the previous communication (Bose and Subrahmanyam, 1949, p. 1, *this issue*), it was

thought desirable to study the effectiveness of the anti-oxidants on oils of different acidities, both under dry and wet conditions.

Very little work has been done on the protective action of anti-oxidants in presence of sunlight. Evers (1929) found practically no preservative action of hydroquinone (0.05 per cent) on the vitamin A content of cod-liver oil exposed to light. Doctor and Banerjee (1939) reported that the presence of hydroquinone could not retard the destructive action of light on the vitamin A in ghee. The extent of protection afforded by anti-oxidants to vitamin A in shark-liver oil exposed to diffused and direct sunlight has been studied here under accelerated conditions.

EXPERIMENTAL.

Each of the samples of oils of varying acid values, that were stored in the refrigerator up to the time of experiment, was rigorously dried on shaking with sufficient amount of anhydrous sodium sulphate and filtering the supernatant oil through a column of a fresh amount of anhydrous sodium sulphate under suction. While treating the oil with sodium sulphate, it was maintained at about 18°C. for effective dehydration.

In two cases, from about two parts of an oil of low acidity, the fatty acids were isolated (Rao, 1946) and added to 25 parts of the same sample of oil in order to demonstrate the influence of increased free acidity on the degree of protection afforded by the anti-oxidants to oils of the same origin but of different acidities.

A portion of each of the dried oils was shaken up thoroughly with 0.5 per cent. (by volume) distilled water. In order to study the combined influence of different levels of moisture and varying acidities on the anti-oxxygenic action, portions of each of the three representative samples of dried oils of acid values 0.54, 5.18 and 14.36 were intimately mixed with 1.0 per cent and 2.0 per cent water (by volume) which are the usual moisture contents of commercial samples.

Half of each of these dried and moist oils was treated with anti-oxidants (0.02 per cent iso-butyl gallate + 0.01 per cent citric acid) by trituration in a mortar and the other halves were used as control samples. One c.c. of each of these control and protected oils was stored at 40°C. in a number of clean and dry bottles (50 c.c.) fitted with rubber-stoppers and sealed with rosin-wax. At regular intervals, three bottles of each set were taken out, the oils contained in them were mixed together and vitamin A in the dry samples as well as in the samples containing 0.5 per cent water was estimated by the Pulf-rich photometer method with filter S.61 (Rao, 1944). Since the Carr-Price reaction may be inhibited in the case of the samples containing 1.0 or 2.0 per cent water, their vitamin A contents were estimated by the Hilger vitameter A method (Vitamin Assay Committee of the American Drug Manufacturers' Association, 1937). The induction periods were determined from the curves drawn with time in hours as abscissæ and loss in vitamin A (per cent) as ordinates. Protection factor was calculated as reported before (Bosé and Banerjee, *loc. cit.*). The results are summarized in Table I and the important curves are shown in Graphs 1 and 2.

TABLE I.

Keeping quality of dry and wet samples of shark-liver oils of varying acid values treated with and without anti-oxidants.

Sample of oil.	Acid value.	INDUCTION PERIOD (HOURS).		Protection factor.
		Control oil.	Oil + iso-butyl gallate (0.02%) + citric acid (0.01%).	
A dry ...	0.54	68	1,034	14.2
A moist *	66	970	13.7
A + 1.0% water	62	750	11.1
A + 2.0% water	62	744	11.0
A + acids, dry ...	10.87	60	522	7.7
A + acids, moist	55	396	6.2
B dry ...	5.18	56	554	8.9
B moist	52	452	7.7
B + 1.0% water	48	254	4.3
B + 2.0% water	46	238	4.2
C dry ...	0.81	72	1,116	14.5
C moist	70	1,042	13.9
C + acids, dry ...	12.02	62	528	7.5
C + acids, moist	56	392	6.0
D dry ...	2.95	58	644	10.1
D moist	55	562	9.2
E dry ...	1.32	64	826	11.9
E moist	61	744	11.2
F dry ...	0.98	68	1,006	13.8
F moist	66	938	13.2
G dry ...	1.86	62	750	11.1
G moist	58	662	10.4

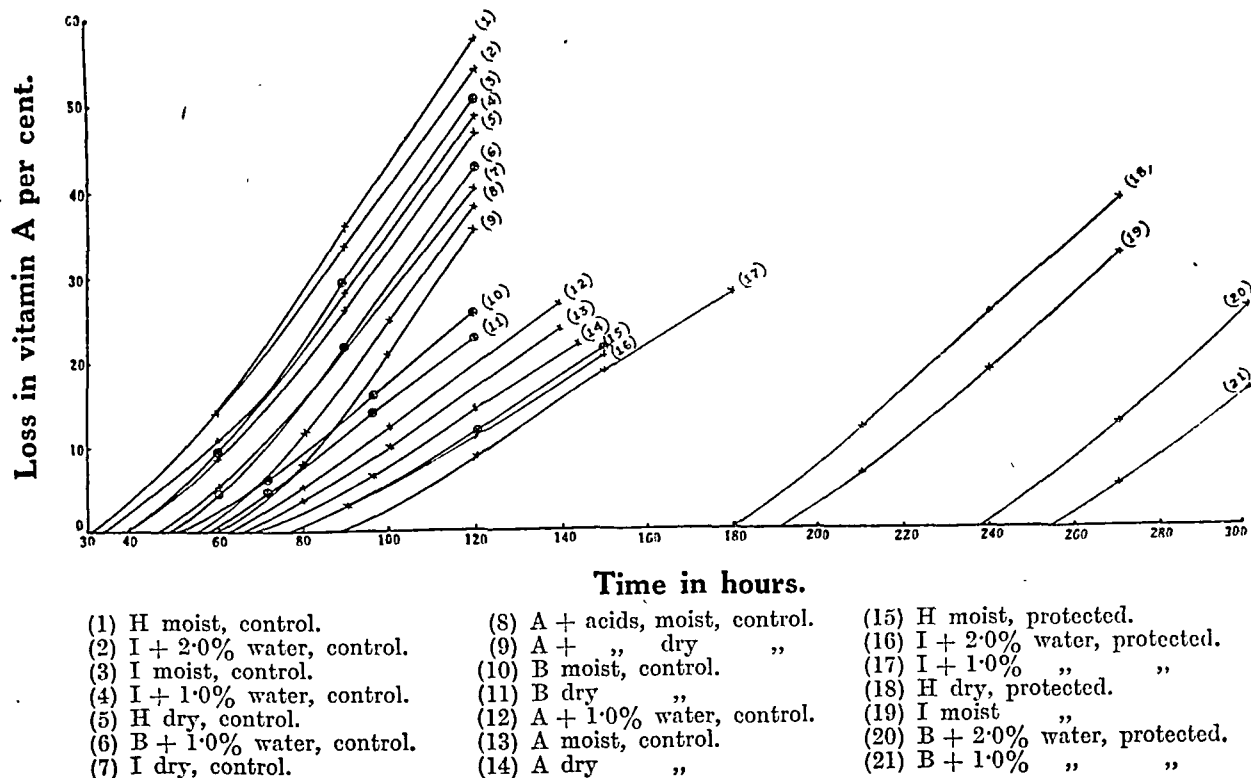
* The word 'moist' stands for 0.5 per cent water (by volume).

TABLE I—concl'd.

Sample of oil.	Acid value.	INDUCTION PERIOD (HOURS).		Protection factor.
		Control oil.	Oil + iso-butyl gallate (0.02%) + citric acid (0.01%).	
H dry	19.37	38	178	3.7
H moist	30	78	1.6
I dry	14.36	44	304	5.9
I moist	38	190	4.0
I + 1.0% water	32	90	1.8
I + 2.0% water	30	78	1.6

GRAPH 1.

Effect of acidity and moisture on the keeping quality of control and protected oils.

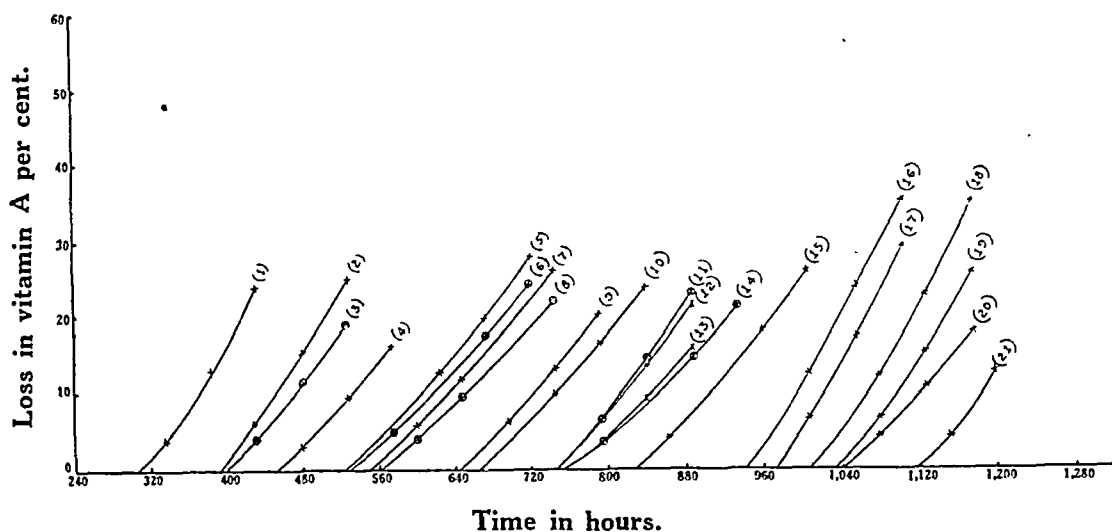


The word 'moist' stands for 0.5% water (by volume).

In order to study the influence of diffused sunlight on the protection of vitamin A by anti-oxidants, 2 c.c. samples of shark-liver oil (acid value 0.68), treated with and without anti-oxidants (0.02 per cent iso-butyl gallate + 0.01 per cent citric acid), were spread out into a number of closed Petri-dishes (7.5 cm. diameter). The first batch of dishes containing both control and treated oils were stored in a dark

GRAPH 2.

Effect of acidity and moisture on the protection of vitamin A by anti-oxidants.



- | | | |
|----------------------------------|-------------------------------|------------------------|
| (1) I dry, protected. | (8) D moist, protected. | (15) E dry, protected. |
| (2) C + acids, moist, protected. | (9) D dry " | (16) F moist " |
| (3) A + " " " | (10) G moist " | (17) A " " |
| (4) B moist, protected. " | (11) A + 2% water, protected. | (18) F dry, protected. |
| (5) A + acids, dry protected. | (12) E moist, protected. | (19) A " " |
| (6) C + " " " | (13) A + 1% water, protected. | (20) C moist " |
| (7) B dry, protected. " | (14) G dry, protected. | (21) C dry " |

The word 'moist' stands for 0.5% water (by volume).

cupboard at room temperature (30°C. to 35°C.). The second batch containing similar samples were stored in diffused light of the laboratory room. At regular intervals, two dishes from each set were taken, the oils contained in them were mixed together and vitamin A was estimated by the Pulfrich photometer method as before. The results are given in Table II.

TABLE II.

Destruction of vitamin A in control and protected oils stored in darkness and diffused light.

Period of storage hours.	CONTROL SAMPLES STORED IN :			
	DARKNESS.		DIFFUSED LIGHT.	
	Vitamin A B 1% 1 cm.	Loss in vitamin A per cent.	Vitamin A B 1% 1 cm.	Loss in vitamin A per cent.
0	8.02	0	8.02	0
60	8.02	0	8.02	0
120	7.74	3.5	7.32	8.8
180	7.04	12.2	6.79	15.4
240	5.95	25.8	5.43	32.3

	PROTECTED SAMPLES STORED IN :			
0	8.02	0	8.02	0
1,080	8.02	0	8.02	0
1,200	8.02	0	6.98	13.0
1,320	8.02	0	6.43	19.8
1,440	6.87	14.4
1,560	5.43	32.3	3.58	55.4
1,680	3.97	50.5

For studying the influence of direct sunlight, a third batch of open Petri-dishes containing 2 c.c. samples of the control and protected oils were exposed to direct sunlight between 10 a.m. and 2 p.m. The temperature of the exposed oils varied between 35°C. and 40°C. during the course of the experiment. Every one hour, two dishes from each set were transferred to a dark cupboard, the oils contained

were mixed together and vitamin A was estimated as before with the least possible delay. The results are given in Table III :—

TABLE III.

Destruction of vitamin A in control and protected oils exposed to direct sunlight.

Period of storage hours.	CONTROL.		PROTECTED.	
	Vitamin A B 1% 1 cm.	Loss in vitamin A per cent.	Vitamin A B 1% 1 cm.	Loss in vitamin A per cent.
0	8.02	0	8.02	0
1	6.35	20.8	6.98	13.0
2	3.80	52.6	3.97	50.5
3	2.74	65.9	3.32	58.6
4	2.01	74.9	2.50	68.8

DISCUSSION.

The results given in Table I show that high free acidity not only adversely affects the keeping quality of the unprotected oils, but also lowers considerably the efficiency of the added anti-oxidants. The adverse effect of moisture is more prominent against the oils of high acidity than against those of low acidity. The combined catalytic influence of increased free acidity and moisture content appears to destroy the protective power of the anti-oxidants studied. The destructive power of 1.0 per cent water is consistently higher than that of 0.5 per cent. There seems to be, however, no marked difference between the effects of 1.0 and 2.0 per cent water content on the storage property of the control oils as well as on the protective power of the anti-oxidants. These observations are of obvious importance from the point of view of the practical applicability of anti-oxidants. Freshly extracted, moisture-free oils of low acid values only (preferably below 1.0) respond to the action of the anti-oxidants studied.

The results given in Table III show that the destruction of vitamin A in shark-liver oil exposed to direct sunlight in thin layers is enormous and the anti-oxidants added are incapable of retarding the destructive action of direct sunlight.

The protective power of the anti-oxidants is found to be partly reduced in presence of diffused light. The difference between the rates of destruction of vitamin A in the control samples stored in diffused light and those stored in darkness, however, is not considerable. The apparent difference in the rates of destruction of vitamin A in shark-liver oil exposed to direct sunlight and those in diffused light

may arise from the fact that, in the latter case, most of the ultra-violet rays which are known to be powerful agents causing destruction of vitamin A are cut off in the process of transmission through thick glass-panes of the windows and covers of the Petri-dishes. The higher rate of destruction in direct sunlight in comparison with that in diffused light may be partly accounted for by the difference in the temperature of the oils subjected to diffused light and direct sunlight, the difference, in some cases, being nearly 10°C. under the experimental conditions.

SUMMARY.

1. The influence of acidity and moisture and of diffused and direct sunlight on the protection of vitamin A in shark-liver oil by anti-oxidants (0.02 per cent iso-butyl gallate + 0.01 per cent citric acid) has been studied.

2. The high free acidity was found to lower considerably the efficiency of the added anti-oxidants. The combined influence of increased free acidity and moisture content was found to destroy the protective power of the anti-oxidants studied.

3. The added anti-oxidants could not retard the destructive action of direct sunlight. In presence of diffused light, the protective power of the anti-oxidants was only partly reduced.

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ANALYSES OF SOUTH INDIAN FOOD PREPARATIONS.

BY

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INTRODUCTION AND GENERAL PROCEDURE.

ANALYSES of common South Indian food preparations are needed by dietitians and doctors in planning diets to meet the requirements of individuals or groups. Accordingly, a beginning has been made with some of the common food preparations. Although there have been no standardized recipes or standardized methods of preparations in South India, the ingredients used in a common preparation are the same with perhaps variations in proportions.

Hotel samples were selected to represent foods consumed by a large number of people and other samples were prepared in the laboratory where the ingredients were carefully measured.

Analyses of moisture, protein, fat, total ash, calcium, phosphorus, iron and calories were made (a) from four samples of each preparation purchased from different hotels in Madras and (b) from one sample prepared in the laboratory (called 'home-made' sample). Two analyses of each sample were made. Since the hotel samples were found to differ from the controlled laboratory samples, the results are given separately. These analyses were made between June 1944 and June 1946.

ANALYTICAL PROCEDURE.

The food preparation was weighed as purchased or made and the dimensions, where possible, noted. Of the sample, 200 to 300 grammes were then ground to a homogeneous mass and fractions of this were used for each determination.

1. *Moisture*.—About 5 to 10 grammes of the sample were dried to constant weight in an electric oven at 120°C. and the loss in weight was taken as the moisture content.

* Work done under the inquiry of the Indian Research Fund Association on Poor Rice Diets and Food Preparations in common use in South India under Dr. (Miss) Eleanor D. Mason at the Women's Christian College, Madras. Grateful acknowledgment is made of grants from the Indian Research Fund Association, from the Elizabeth Thompson Science Fund, Boston, U.S.A., also of the guidance of Dr. Eleanor D. Mason and Miss Dorothy M. Pearson of the Department of Physiology and Nutrition, Women's Christian College, Madras.

2. *Protein*.—The total nitrogen in 1 to 2 grammes of the sample was determined by the macro-Kjeldahl method and the protein content calculated by multiplying the grammes of nitrogen by 6.25.

3. *Fat*.—Ether extractives (Soxhlet apparatus) in 20 to 30 grammes of the sample were reckoned as fat.

4. *Total ash: mineral matter*.—Ten to fifteen grammes of the sample were dried and burnt over a Bunsen flame in a platinum or silica dish and ashed to constant weight. The weight of the ash was taken as the total mineral matter.

5. *Fractional analysis of the ash*.—Ash obtained as above for 100 grammes was moistened with a few drops of distilled water and 5 c.c. to 6 c.c. of iron-free concentrated hydrochloric acid were added. The solution was boiled, cooled and diluted with distilled water and filtered into a 100 c.c. measuring flask. The filtrate and washings of the filter-paper were made up to mark and aliquots were taken for the following determinations:—

(a) *Calcium*.—Twenty c.c. of the above solution were made alkaline with ammonia, and excess of potassium oxalate was added and the solution boiled. While hot, it was made acid with strong acetic acid. The precipitate of calcium oxalate was allowed to stand overnight. It was then washed free from chlorides with boiling distilled water through Whatman No. 44 filter-paper, dissolved in dilute sulphuric acid and titrated against deci-normal potassium-permanganate solution.

(b) *Phosphorus*.—To 20 c.c. of the ash solution, excess of crystals of potassium nitrate was added and the solution warmed in a water-bath. Ammonium-molybdate solution was added little by little with constant stirring. When the precipitation of the phospho-molybdate was complete, it was allowed to settle overnight, washed with 3 per cent potassium-nitrate solution through Whatman No. 44 filter-paper till free from acid. It was then dissolved in a known volume of standard sodium-hydroxide solution and the excess titrated against standard nitric acid.

(c) *Iron*.—Iron was estimated colorimetrically by the potassium-thiocyanate method, the colour being extracted by pure amyl alcohol.

6. *Total calories*.—Calories were determined directly by the use of Benedict and Fox's (1925) apparatus known as the Benedict-Fox oxy-calorimeter. About 5 grammes of the sample were dried in the electric oven and powdered, and 1 to 2 grammes of the powder accurately weighed was ignited electrically and burnt in a closed circuit in an atmosphere of oxygen. The volume of oxygen used up for the combustion was noted at constant temperature. This volume was corrected for any unburnt carbon in the ash (which was never more than a few milligrams) for the nitrogen liberated, and finally corrected to N.T.P. The corrected volume in litres was multiplied by the factor 4.825 (calorific value per litre oxygen for mixed foods) and thus the calories computed. This method has the advantage of measuring the calorific value of the food directly without full analysis of and subsequent calculation from the protein, fat and carbohydrate contents.

TABLE I.
Recipes and methods of cooking 'home-made' sample.

Number.	Preparation.	INGREDIENTS.			Method.	Amount.
		Name of ingredient.	Measure.	Weight in g.		
1	Puri	Whole-wheat flour (atta)	2 ollocks*	250	Atta, salt and water were mixed well and small balls were made and rolled and fried in hot deep ghee.	14 puris. Total weight: 370 grammes.
		Common salt	2 teaspoonfuls	5		
		Water	3/4 ollock	147		
		Ghee used in frying†	...	49		
2	Vadai	Black gram without outer husk.	2 ollocks	350	Black gram was soaked in water for 2 hours, then cleaned, drained and ground finely. The other ingredients were chopped and added. Small portions were flattened by hand and fried in hot deep oil and removed when brown.	23 vadais. Total weight: 666 grammes.
		Green chillies	12 medium	25		
		Onion	1 big	70		
		Ginger	1 small piece	9		
		Curry leaves	Few	5		
		Coriander leaves	Few	6		
		Common salt	4 teaspoonfuls	13		
		Ground-nut oil used in frying.	...	53		

* All measurements are levelled. An ollock is one-eighth of a Madras measure which is the standard measure in South India. The capacity of an ollock is about 190 c.c.
 † Ghee or oil used in frying, in this column is the difference between the weights of the oil before frying and after frying. Therefore this does not take into account the oil wasted in handling or adhering to the sides of the vessels.

TABLE I—*contd.*

Number.	Preparation.	INGREDIENTS.			Method.	Amount.
		Name of ingredient.	Measure.	Weight in g.		
3	Uppuma	Whole-wheat broken as 'ravai'.	1 ollock	150	To the oil heated to boil in the pan, mustard, black gram, cashew-nuts, chopped green chillies, onion, curry leaves and ginger were added in the order mentioned and fried. Then water and salt were added and when the water boiled, 'ravai' was added with constant stirring until cooked to a solid mass.	6 plates. Total weight: 615 grammes.
		Black gram dhal	3 teaspoonfuls	14		
		Cashew-nuts	...	25		
		Mustard	$\frac{1}{2}$ teaspoonful	4		
		Curry leaves	Few	2		
		Onion	$\frac{1}{2}$ big	40		
		Green chillies	3 medium	5		
		Ginger	1 small piece	2		
		Common salt	3 teaspoonfuls	10		
		Gingelly oil	...	90		
		Water	2 ollocks	388		
4	Iddly	Parboiled milled rice	2 ollocks	398	Rice and gram were separately soaked in water for 5 hours; then washed, drained and ground and mixed well with salt and left overnight. The next morning they were steamed in a special steamer called 'iddly pathram'.	20 iddlis. Total weight: 1,262 grammes.
		Black gram dhal	$\frac{1}{2}$ ollock	95		
		Common salt	4 teaspoonfuls	13		
		Water added after soaking	1 $\frac{1}{2}$ ollocks	300		

5	Omappodi	Bengal gram dhal flour ...	4 ollocks	...	395	All the ingredients except oil were mixed to a thick dough and pressed through the mould into hot deep oil and fried and removed when golden brown.	Total weight : 608 grammes.
		Raw rice flour ...	1 ollock	...	147		
6	Dhosai	Onum powder ...	1 pinch	...	2	Rice and gram were separately soaked for 5 hours, then washed, drained and ground and mixed well with salt and left overnight. The next morning the batter was poured on to flat oiled 'dhosai pans' and cooked.	38 dhosais. Total weight : 1,868 grammes.
		Common salt ...	3½ teaspoonfuls	...	12		
		Water ...	1½ ollocks	...	290		
		Gingelly oil used for frying	177		
		Parboiled milled rice ...	4 ollocks	...	710		
7	Pagoda	Black gram dhal ...	1 ollock	...	170	Onion, chillies, ginger curry and coriander leaves were chopped and all the ingredients except oil were mixed. Small bits of the dough were fried in hot deep oil and removed when brown.	Total weight : 220 grammes.
		Common salt ...	6 teaspoonfuls	...	20		
		Gingelly oil used in frying	20		
		Water added after soaking	3½ ollocks (nearly)	...	740		
		Bengal gram dhal flour ...	1 ollock	...	100		
		Raw rice flour ...	½ ollock	...	60	Onion, chillies, ginger curry and coriander leaves were chopped and all the ingredients except oil were mixed. Small bits of the dough were fried in hot deep oil and removed when brown.	Total weight : 220 grammes.
		Onion ...	1 medium	...	60		
		Green chillies	6 medium	...	10		
		Ginger ...	1 small piece	...	5		
		Curry leaves ...	Few	...	2		
		Coriander leaves	Few	...	1		
		Common salt	3 teaspoonfuls	...	9.5		
		Water added	½ ollock (nearly)	...	52		
		Gingelly oil used in frying	78		

TABLE I—*concl'd.*

Number.	Preparation.	INGREDIENTS.			Method.	Amount.
		Name of ingredient.	Measure.	Weight in g.		
8 (a)	Bajji plantain ...	Bengal gram dhal flour ...	$\frac{1}{2}$ ollock	58	All the ingredients except oil and plantain were mixed well and the plantain was sliced into discs which were dipped in the dough and fried in hot deep oil until brown.	23 bajjis. Total weight: 175 grammes.
		Raw rice flour ...	$\frac{1}{2}$ ollock	14		
		Common salt ...	1 teaspoonful	3.5		
		Turmeric ...	1 pinch	1.0		
		Dry chilli powder ...	1 teaspoonful	2.5		
		Soda bicarbonate ...	1 pinch	0.5		
		Asafœtida ...	1 small piece	0.7		
		Water added ...	$\frac{3}{4}$ ollock	75		
		Gingelly oil used ...	"	55		
		Plantain (green) ...	$\frac{3}{4}$ of a medium	61		
8 (b)	Bajji onion ...	The same as above except gingelly oil used.	...	45	Same as above	25 bajjis. Total weight: 200 grammes.
		Onion (instead of plantain)	3 medium	82		
8 (c)	Bajji brinjal ...	The same as above except gingelly oil used.	...	109	Same as above	42 bajjis. Total weight: 230 grammes.
		Brinjal	2 medium	80		

TABLE II.

Analyses of common South Indian food preparations, and comparisons of the values obtained from direct analysis with values calculated from tables (based on weights used in 'home-made samples').*

Number.	Preparation.	Samples.	Average cost.	Average size.	Average weight, grammes.	FOOD CONSTITUENTS.							Calories/100 g.	Iron, mg./100.	Carbohydrate Cal./100 g. ap- proximately.
						Moisture, g./100.	Protein, g./100.	Fat, g./100.	Ash, g./100.	Calcium, g./100.	Phosphorus, g./100.				
1	Puri	Mean of 4 hotels	Rs. a. p. 0 0 9 per puri.	Diameter 10 cm. Thickness 0.5 cm.	17	24.00	8.99	14.36	1.401	0.049	0.120		377	92.4	..
		'Home-made' as analysed.	0 0 5 per puri.	Diameter 12 cm. Thickness 0.5 cm.	27	27.61	9.14	9.49	2.098	0.047	0.075		337	6.1	..
		As calculated	"	27	.. †	(8.17)	(14.39)	(2.567)	(0.027)	(0.216)		(357)	(4.9)	..
2	Vadai	Mean of 4 hotels	0 0 6 per vadai.	Diameter 6 cm. Thickness 2 cm.	24	40.21	12.49	14.79	2.169	0.057	0.082		300	16.9	..
		'Home-made' as analysed.	0 0 3 per vadai.	Diameter 7 cm. Thickness 2 cm.	29	40.58	12.92	6.16	3.878	0.058	0.034		311	11.4	..
		As calculated	"	29	..	(13.31)	(8.77)	(3.933)	(0.136)	(0.210)		(270)	(5.6)	..
3	Uppuma	Mean of 4 hotels	0 1 0 per serving.	About 1 cup ..	67 g./serving.	63.52	2.78	9.85	0.992	0.031	0.043		179	7.8	..
		'Home-made' as analysed.	0 0 9 per-serving.	"	62	57.85	1.18	12.75	1.554	0.072	0.076		252	4.8	..
		As calculated	"	62	..	(4.70)	(17.64)	(2.300)	(0.030)	(0.116)		(247)	(2.0)	..

* The food values were calculated from Health Bulletin No. 23 of Government of India Press, 1941.—The Nutritive Value of Indian Foods and the Planning of Satisfactory Diets.

† Moisture content was not calculated as it was impossible to account for the amount lost in evaporation.

‡ Calculated as difference between total calories and calories from protein (g. × 4) and from fat (g. × 9).

FOOD CONSTITUENTS.

[illegible]

9/4	Bsiji plantain	Mean of 2 hotels	0 0 3 per bajji.	Diameter 4 cm.	12	40.36	7.64	22.46	4.12	0.038	0.072	16.6	181	..
		'Home-made' as analysed.	..	"	7	40.58	5.60	24.16	2.32	0.051	0.152	11.6	236	..
		As calculated	"	7	..	(6.99)	(33.41)	(3.53)	(0.074)	(0.153)	(3.8)	(445)	..
8/5	Bsiji onion	Mean of 2 hotels	0 0 2 per bajji.	Diameter 2 cm.	4	44.4	6.94	21.45	3.009	0.046	0.102	9.8	222	..
		'Home-made' as analysed.	0 0 3 per bajji.	Diameter 3 cm.	8	47.77	5.64	18.49	2.630	0.039	0.114	16.2	202	..
		As calculated	"	8	..	(6.18)	(24.21)	(3.100)	(0.135)	(0.104)	(3.4)	(345)	..
8/2	Bsiji brinjol	Mean of 2 hotels	0 0 3 per bajji.	Diameter 3 cm.	10	46.61	6.46	17.90	2.390	0.047	0.110	10.2	188	..
		As calculated	"	10	..	(5.19)	(48.94)	(2.582)	(0.039)	(0.090)	(3.0)	(534)	..

The apparatus (Benedict-Fox's oxy-calorimeter) consists of a combustion chamber in which the weighed sample is burnt, a soda-lime container for absorption of carbon dioxide, a spirometer for measuring the oxygen used, and a motor-blower for circulating the gases. Thermometers are inserted in the circuit and in the spirometer.

RESULTS.

The recipes and the results of analyses are shown in Tables I and II.

The comparison of the data of the hotel and 'home-made samples' shows the hotel samples markedly greater in cost and smaller in size. Hotel samples of fried preparations in most cases contained more fat and were consequently higher in caloric value. Comparison of the food values obtained from direct analysis and calculation shows that the calculated figures for fat and calories are greater than the value obtained by actual analysis. This is undoubtedly largely due to the fact that while the entire oil used in the preparation is taken into account in calculation, in the actual preparation considerable quantities of oil are lost in handling, adhering to the vessel and left as residue after draining. The total ash value as calculated is also slightly greater than as analysed. This difference may be due to the fact that the weight of the common salt was calculated in entirety as dry mineral regardless of its moisture content. The iron values are greater in the data obtained by direct analysis. The values of phosphorus and calcium are on the whole comparable.

Thus, we see that quite apart from the labour involved in such calculations, the actual analyses are more accurately representative of the food that is eaten. It would be useful to have such analyses made on more of the common South Indian food preparations and on preparations from other parts of India.

SUMMARY.

1. Analyses of moisture, protein, fat, total ash, calcium, phosphorus, iron and calories were made on the following South Indian food preparations for the use of dietitians and doctors planning diets: puri, vadai, uppuma, iddly, omapodi, dhosai, pagoda and bajji.

2. The analyses were made from four hotel samples and one 'home-made' sample and the hotel and 'home-made' samples compared.

3. The values obtained from the analyses are compared with the values calculated from tables for the raw ingredients used in the preparation.

4. The values from direct analyses of foods as actually eaten indicate that the use in dietary studies of calculated values for food preparations is likely to give misleading figures, particularly with regard to fat and total calories.

REFERENCE.

BENEDICT, F. G., and FOX, E. L. *Jour. Biol. Chem.*, **66**, p. 783. (1925).

ANALYSES OF SOME EDIBLE GREEN LEAVES IN SOUTH INDIA.

BY

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INTRODUCTION.

It is well known that the poorer classes in South India include in their diet a large variety of green leafy plants found growing among the grass and weeds. Eight different kinds of such 'greens', which are not found among those reported from Coonoor under 'Green Leafy Vegetables' in Health Bulletin No. 23 (1941), were collected, identified and analysed. The analysis of one of these (*Trianthema monogyna*) is given by Nicholls (1945) in his work on Tropical Nutrition which includes several varieties of 'greens' used in the tropics. Besides these eight, two others—'paruppu keerai' (*Portulaca olearacea*) and radish tops (*Raphanus sativus*)—were also analysed. The analysis of *Raphanus sativus* is also given by Nicholls in the above-mentioned work, while its vitamin C content has been determined by Ahmad (1935).

METHOD OF INVESTIGATION.

Collection of greens.—All 'greens' with the exception of 'paruppu keerai' and radish tops were collected from among the weeds in the compound of the Women's Christian College, the two samples being collected from different parts

* Work done in part under the Inquiry on Poor Rice Diet and Food Preparations in Common Use in South India of the I.R.F.A. under Dr. (Miss) Eleanor D. Mason, Women's Christian College, Madras. Grateful acknowledgment is made of grants from the Indian Research Fund Association, and from the Elizabeth Thompson Science Fund, Boston, U.S.A.

of the compound. Only edible portions were used—thorns in 'nerringi', flowers and pods of 'vella keerai', and flowers of 'kuppameni' and 'kuppa keerai' being discarded.

Methods used in chemical analysis.—Two analyses were made of each sample. For protein and vitamin C estimations the fresh leaves were used, while the rest of the sample was accurately weighed and dried in the oven at 80°C. to 90°C. for ten to twelve hours. The dried sample was powdered and stored away from light in glass-stoppered bottles. Weighed samples of this powder were taken for total ash, calcium, phosphorus, iron, caloric and fat estimations. The values were then re-calculated for fresh weight.

The protein content was determined by the macro-Kjeldahl method. Calcium was precipitated as calcium oxalate which was titrated against standardized 0.1 N potassium permanganate in presence of excess dilute sulphuric acid. Phosphorus was estimated by precipitating as ammonium phospho-molybdate. Iron was estimated by extracting the red colour obtained when potassium thiocyanate is added to the ash solution, with amyl alcohol, and comparing the depth of colour with a standard iron solution in the calorimeter. The caloric value was determined by the apparatus oxy-calorimeter as devised by Benedict and Fox (1925). The reduction method using 2.6 dichlorophenol-indophenol dye was used for vitamin C estimation.

DISCUSSION.

From the analysis it may be seen that many of the leaves are rich in either minerals or vitamin C or in both. They are thus valuable cheap supplements to diet which in South India are predominantly rice. Further, the analysis shows that *Tribulus terrestris* is rich in calcium and poor in iron, while *Trianthema monogyna* is poor in calcium and rich in iron. Here the compensatory value of mixed 'greens' is seen. The poorer classes appear to have learned this by experience, for, as a rule, they use a mixture of 'greens' (kalavai keerai) rather than a single 'green' by itself.

These leaves are not available in the market but are freshly picked for use by the common people, often by the roadside. This saves losses in vitamin content otherwise caused by wilting and exposure as in the local markets. It may be clearly seen that these 'greens' supplement in more than one way the miserably inadequate diet of the poor city dwellers. It is interesting that one of them, *Tribulus terrestris*, is included in the list of famine herbals of China (Read, 1946). Besides these which have been analysed, there are several others which are commonly used. These need to be identified, analysed, and their cultivation encouraged.

SUMMARY.

1. Eight different kinds of green leafy herbs found growing among the weeds of the compound and commonly used by the poorer classes in Madras city were collected, identified and analysed for moisture, protein, fat, total ash, calcium, phosphorus, iron, calories and vitamin C.

2. They were found to be rich in calcium, iron and vitamin C (analyses given in the Table).

PLATE I.

Green leafy vegetables used by people in Madras city.

(Drawn slightly smaller than natural size.)

Thanks are due to Miss Anna Zachariah, M.Sc., of the Women's Christian College, for the identification of the plants (Figs. 1 to 8).

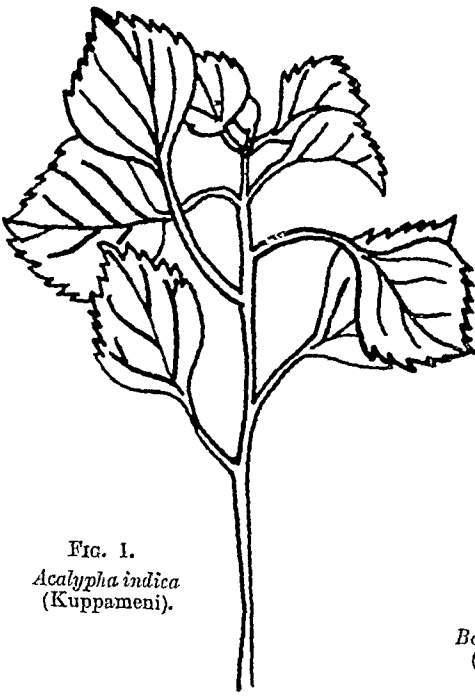


FIG. 1.
Acalypha indica
(Kuppameni).

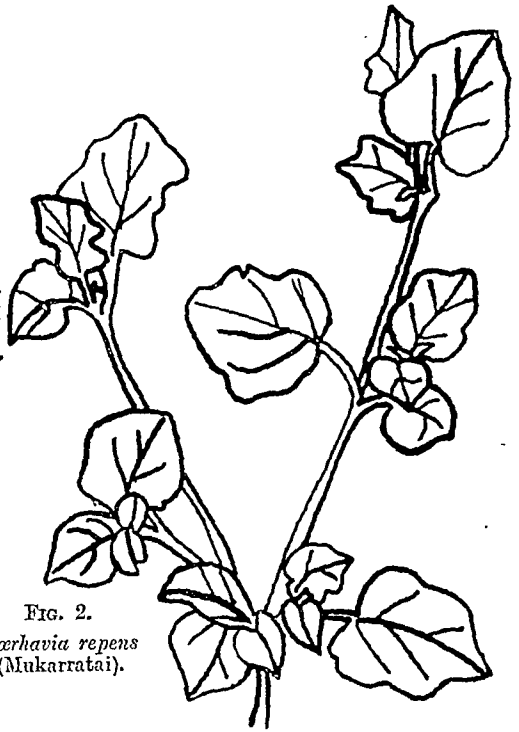


FIG. 2.
Barhavia repens
(Mukarratai).

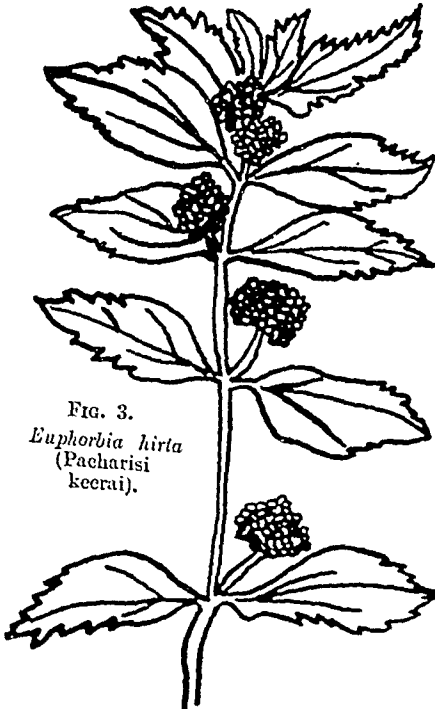


FIG. 3.
Euphorbia hirta
(Pacharisi
keerai).

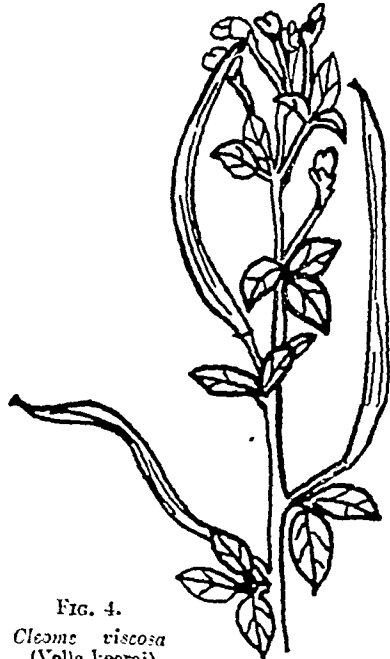


FIG. 4.
Cleome viscosa
(Vella keerai).

PLATE II.

Green leafy vegetables used by people in Madras city.

(Drawn slightly smaller than natural size.)

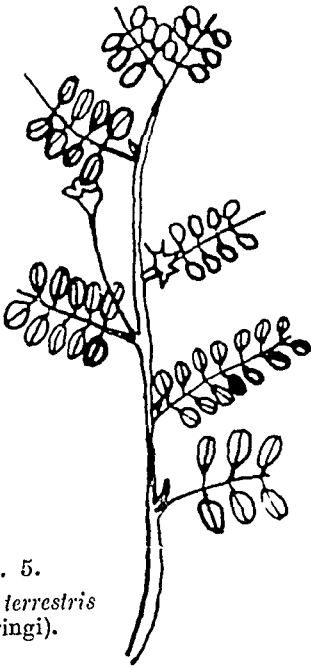


FIG. 5.
Tribulus terrestris
(Nerringi).

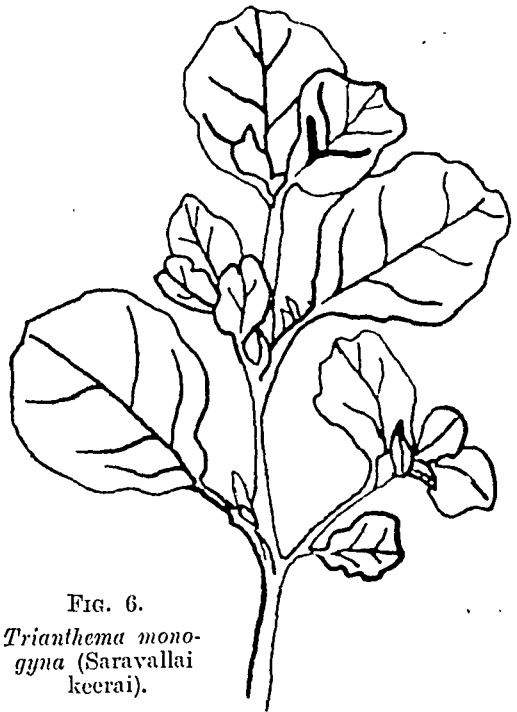


FIG. 6.
Trianthema mono-
gyna (Saravallai
keelai).

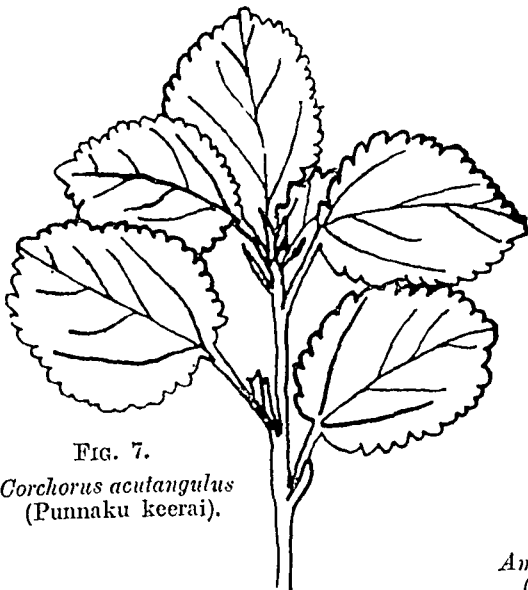


FIG. 7.
Corchorus acutangulus
(Punnaku keelai).



FIG. 8.
Amaranthus viridis
(Kuppa keelai).

TABLE.
Analyses of green leafy vegetables used by the common people in Madras city.
(Value per 100 g.)

Tamil name.	Botanical name.	Moisture, g.	Protein, g.	*Ether extrac- tives, g.	Total ash, g.	Calcium, g.	Phospho- rus, g.	Iron, mg.	Calories.	Vitamin C, mg.
Paruppu keerai ...	<i>Portulaca oleracea</i> ...	94.11	1.67	...	2.408	0.084	0.051	25.09	35	26.03
Radish tops ...	<i>Raphanus sativus</i> ...	90.37	2.43	...	2.295	0.289	0.039	17.83	46	119.5
Kuppameni ...	<i>Acalypha indica</i> ...	80.54	6.74	1.41	3.14	0.667	0.99	17.31	64	147.9
Mukarratai ...	<i>Barbarea repens</i> ...	84.53	6.09	0.90	4.31	0.667	0.069	18.39	65	27.23
Pachariki keerai ...	<i>Euphorbia hirta</i> ...	78.14	4.65	1.71	3.15	0.546	0.106	21.24	120	44.32
Vella keerai ...	<i>Cleome viscosa</i> ...	80.41	5.64	1.85	3.75	0.881	0.073	24.45	93	†203.6
Neringi ...	<i>Tribulus terrestris</i> ...	79.09	7.22	0.541	4.63	1.55	0.082	9.22	93	41.53
Saravallai keerai ...	<i>Trianthema monogyna</i>	91.33	2.01	0.390	2.62	0.056	0.03	38.54	36	†69.60
Punnaku keerai ...	<i>Corchorus acutangulus</i>	80.60	6.12	0.67	2.51	0.25	0.038	35.65	48	†145.9
Kuppa keerai ...	<i>Amaranthus viridis</i> ...	81.75	5.17	0.33	2.84	0.33	0.052	18.74	38	†178.0

* Ether extractive includes chlorophyll.

† Vitamin C estimation done on different sample.

the growth response and fertility of rats reared exclusively on a poor South Indian rice diet. Some interesting results obtained in the course of the above investigation are recorded in this paper.

EXPERIMENTAL.

The poor South Indian rice diet as used by us had the following percentage composition :—

Polished rice, 78·5; thur dhal (*Cajanus indicus*), 5·0; common salt, 0·3; non-leafy vegetables, 8·2; leafy vegetables, 2·1; whole milk powder, 0·9; and fat or oil, 5·0.

The rice was washed twice prior to cooking. The washed rice, dhal and vegetables were cooked together with roughly three times the volume of water and thoroughly mixed with the fat which was previously heated to 180°C. and maintained at that temperature for five minutes to simulate the common practice in frying. The milk powder was separately fed as a 10 per cent solution.

The experimental animals were weaned on the 28th day and maintained on the particular diet to be tried *plus* 10 c.c. of 10 per cent whole milk powder for a week after which the extra milk was stopped. The weights of the rats after this initial feeding for one week were considered as the starting weights for the experiment. This preliminary feeding with milk and the rice diet was found essential not only to accustom the rats to the poor rice diet but also to sustain them over the fairly long period of experimentation.

Experiment I. Supplementation of rice diet with calcium and vitamins at sub-optimal levels.

Young rats were selected when they were 28 days old and divided into 10 groups of 12 each, keeping, as far as possible, the same number of litter-mates of the two sexes in each group. The first five groups received the basal rice diet *plus* 5 per cent each of crude ground-nut oil, refined ground-nut oil, Vanaspati of m.p. 37°C., Vanaspati of m.p. 41°C. and cow ghee, respectively. The second set of five groups received the same basal diet with supplements so that each rat received (i) 30 mg. of calcium lactate per day, (ii) 1 tablet of Squibb's yeast per week (containing 0·06 mg. thiamine, 0·03 mg. riboflavin and 0·15 mg. niacin), and (iii) 1 mg. of mixed tocopherols per week given as a wheat-germ oil concentrate. In addition to these, the proportion of leafy and non-leafy vegetables were adjusted so as to make them 5 per cent in each case.

Weekly records of the weights of rats as well as their daily food intake were maintained for a period of 12 weeks after which the males and females in each group were allowed to mate. Observations of the conditions of the skin and eyes of the animals were made once a week. When any animal died before the conclusion of the experiment, it was dissected and examined. After the normal gestation period, the males were sacrificed and their livers examined for vitamins as also for fat infiltration. Two months after parturition, the females were also killed and their livers examined as above.

The growth rates of the animals in the two series are given in Table I :—

TABLE I.

Growth rates and food intakes of rats on a basal poor rice diet and the same supplemented with calcium and vitamins at sub-optimal levels.

Group.	Fat or oil in the diet.	Number of animals in each group.	AVERAGE VALUES OF :			
			Initial weight in g.	Final weight in g.	Weekly increase in g.	Daily intake of food in g.
	<i>Basal diet :</i>					
A	Crude ground-nut oil ...	6 M	39.0	70.8	2.7 ± 0.2*	7.3
		6 F	38.7	64.7	2.4 ± 0.1	7.3
B	Refined ground-nut oil	6 M	38.5	71.7	2.9 ± 0.2	7.1
		6 F	39.1	64.2	2.1 + 0.0	7.1
C	Vanaspati 37°C. m.p. ...	6 M	37.9	80.1	3.3 ± 0.2	7.1
		6 F	38.2	75.3	3.1 ± 0.1	7.2
D	Vanaspati 41°C. m.p. ...	6 M	38.2	74.2	3.1 ± 0.2	7.2
		6 F	38.1	72.3	2.9 ± 0.1	7.2
E	Cow ghee ...	6 M	39.2	79.8	3.3 ± 0.2	7.3
		6 F	38.6	71.3	2.8 ± 0.1	7.2
	<i>Modified basal diet :</i>					
A	Crude ground-nut oil ...	6 M	38.8	107.8	5.8 ± 0.3*	7.4
		6 F	38.8	102.1	5.3 ± 0.3	7.6
B	Refined ground-nut oil	6 M	39.3	117.2	6.5 ± 0.3	7.5
		6 F	38.5	104.2	5.5 ± 0.3	7.5
C	Vanaspati 37°C. m.p. ...	6 M	37.9	109.5	6.0 ± 0.3	7.5
		6 F	39.3	99.0	5.0 ± 0.3	7.5
D	Vanaspati 41°C. m.p. ...	6 M	38.5	108.3	5.4 ± 0.5	7.6
		6 F	39.9	104.8	4.8 ± 0.3	7.6
E	Cow ghee ...	6 M	38.3	113.6	6.4 ± 0.0	7.6
		6 F	39.7	96.0	4.7 ± 0.2	7.6

* Standard error of the mean.

Many of the rats in the control series did not survive the experimental period. The breeding of the surviving rats was not successful. The animals in the experimental series bred successfully although lactation was very poor. Most of the young ones either died within three days after birth or were eaten up by the mothers. This was found to be the case in all the five groups.

Record of symptoms.

Control series.—The rats were apparently normal till the fifth week. After this, undue dryness of the skin was a constant feature in most of the rats. Shedding of hair commenced at this stage. Complete de-coating occurred only in two animals of the Vanaspati group and these died subsequently. Patches formed by partial de-coating at places were covered up towards the eighth or ninth weeks. Actual lesion of the skin was observed only in one animal in the Vanaspati group. The conditions of the tail and paw were normal in all cases. No opacity or exorcitiation could be observed in the eyes of any animal. Irrespective of the source of fat, the animals in general were far from alert and had a preference for the dark. Some of them developed body twitters described in literature as characteristic of B-complex deficiency. The general conditions of the animals would also suggest poor liver function.

More interesting were the observations made by post-mortem examination of the rats of this series. The stomach was found to be slightly ulcerated and the pyloric end congested and inflamed. The liver had a flabby appearance and was soft to the touch. No macroscopic indication of fat infiltration was observed in any case. The most striking change was noticed in the intestine, the entire length of which had an injected appearance and signs of sub-mucal or capillary hæmorrhage. Further, the mucous membranes had a tendency to peel off as a cast. Kidneys and spleen were normal. There was no sign of atrophy of the regenerative organs although some had a vascular appearance. In certain cases, the lungs were consolidated, indicating that broncho-pneumonia was possibly the terminal condition of the rats.

Experimental series.—In contrast to the animals in the control series, those in the experimental one receiving the sub-optimal supplements of calcium and vitamins did not show any gross sign of de-coating or skin lesion. Dry skin and rough fur were, however, common. In general, the rats were more healthy than those in the control series, although they did not compare with the stock rats.

Examination of the liver.—The method suggested by Lemely, Brown, Bird and Emmert (1947) was followed to extract the unsaponifiable matter in the liver. The Carr-Price reaction was uniformly negative in all the animals of both the series indicating absence of vitamin A in the liver. The fat contents of the livers were within the normal ranges (4 to 5 per cent on fresh weight of liver).

Experiment II. Supplementation of the rice diet with tamarind and chilli.

In a preliminary communication we have already drawn attention to the increase of over 25 per cent in the growth of rats receiving tamarind and chilli supplement in their diet (Krishna Murti, De and Subrahmanyam, 1948). In order

to ascertain the factor or factors responsible for the stimulation of growth by these apparently unimportant dietary constituents the following experiment was carried out :—

Thirty-six young male rats were chosen from our stock colony and divided into six groups. The diets given to the different groups were as follows :—

GROUP I. The basal rice diet without fat. In place of fat, 5 per cent more of rice was included.

GROUP II. Basal rice diet without fat. In place of fat, a soup made up of tamarind, chilli and salt in the proportion of 2 : 1 : 1 by adding 5 per cent to the total solids, was added.

GROUP III. The basal rice diet + 5 per cent Vanaspati of 41°C. m.p.

GROUP IV. Basal rice diet + 5 per cent Vanaspati + 5 per cent tamarind and chilli soup (replacing 5 per cent of rice).

GROUP V. Basal rice diet + 10 per cent Vanaspati of 41°C. m.p.

GROUP VI. Basal rice diet + 10 per cent Vanaspati + 5 per cent tamarind and chilli soup (the extra fat and tamarind soup were made by replacing equal amounts of rice).

Preparation of the tamarind, chilli and salt soup.—Fully ripe tamarind without the rind and the seeds were taken, extracted with water a number of times till all the soluble constituents were dissolved out. Salt and chilli (dry) powder were added in equal proportion to the extract and boiled down to the consistency of a thick syrup. Weighed amounts of this soup to correspond to 5 per cent dry matter made up of tamarind, chilli and salt in the proportion of 2 : 1 : 1 were mixed with the cooked diet prior to feeding.

The growth rates and symptomatic conditions of the animals were observed over a period of six weeks. The results are summarized in Table II :—

TABLE II.

Effect of supplementation with tamarind and chilli.

(Average figures.)

Group.	Number of animals.	Initial weight in g.	Final weight in g.	Weekly growth in g.
I	6	40.3	76.1	6.0 ± 0.2
II	6	40.4	84.2	7.3 ± 0.3
III	6	38.3	67.8	4.9 ± 0.2
IV	6	40.8	77.2	6.1 ± 0.2
V	6	40.2	52.8	2.1 ± 0.1
VI	6	40.7	61.3	3.4 ± 0.2

Symptomatic conditions.—A very striking difference observed in the symptomatic conditions between the animals of the control and experimental groups was with regard to shedding of hair and the roughness of the coat. Depilation was very common in animals of the control group, whereas the animals receiving the tamarind and chilli supplements maintained a smooth coat and not a single animal manifested any signs of alopecia.

DISCUSSION.

The multi-deficient character of the poor rice diet of South India has been discussed by Aykroyd and Krishnan (*loc. cit.*) and Aykroyd, Krishnan, Passmore and Sundararajan (1940) who emphasize deficiencies of vitamin A, various members of B₂ complex and calcium. The rice diet also does not provide the animal body with its basic requirements of the essential dietary factors. The daily intakes of protein and calcium in our experiments were found to be between 0.5 g. to 0.6 g. and 8 mg. to 10 mg., respectively, whereas the adequate requirements of these two factors for optimal growth and proper reproduction have been estimated to be 3.0 g. to 3.5 g. and 40 mg. to 50 mg. respectively (Griffiths and Farris, 1942).

It is clear from the results of experiment I that supplementation of the poor rice diet even at sub-optimal levels with calcium and vitamins has a beneficial effect on the growth and symptomatic conditions of the rats. The supplements, however, have not helped the animals either to breed successfully or lactate the young ones. In a subsequent experiment we have found that the growth and lactation of animals improved considerably when the rice diet was supplemented with 7 per cent casein (fat and vitamin free). Supplementation with calcium carbonate at 0.3 per cent level or with optimal amounts of vitamins (60 I.U. of vitamin A per rat per day, 10 I.U. of vitamin D, 0.5 mg. of α -tocopherol acetate, 0.06 mg. thiamine, 0.03 mg. of riboflavin and 0.15 mg. of niacin) alone did not bring about this improvement in lactation. Based on growth observation, Aykroyd *et al.* (*loc. cit.*) suggested that calcium may be the limiting factor of the diet. Our observations would suggest that protein is an important factor in determining efficiency in reproduction and lactation. This aspect of the subject is now under further study.

It may be seen from the results of experiment II that mere supplementation of the rice diet with tamarind and chilli has a uniformly beneficial effect in all the groups. It is also clear that the supplements have no specific rôle in a better utilization of the fat ingested. The results would also confirm the observations of Mason *et al.* (1946) that on increasing the fat supplements there is a definite inhibition of growth of rats reared on a poor rice diet. Although the animals receiving the tamarind and chilli supplemented diet consumed slightly more of the food given every day, the increase in growth of the animals was out of proportion to the extra amount of food consumed over the control animals. It has already been reported (Krishna Murti, De and Subrahmanyam, *loc. cit.*) that dried tamarind and chilli do not contribute any protein, fat, mineral or vitamins to the diet. The beneficial effect should, therefore, be traceable to other factors. The nature of the agencies responsible for the improved growth are under study and will be the subject of a later communication.

SUMMARY.

1. Supplementation of the poor South Indian rice diet with calcium and vitamins at sub-optimal levels helped to improve the growth of rats from an average of about 3 g. to about 6 g. per week. The animals bred successfully but showed no capacity for lactation. The effect was the same when oil, Vanaspati or ghee was used as the source of fat.

2. Incorporation of tamarind and chilli in the proportions usually added to the South Indian diet improved the growth rate and the general health of the animals. The beneficial effect was small but consistent. Tamarind and chilli appear to have no influence on the utilization of fat.

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SUPPLEMENTARY VALUE OF OIL-SEED CAKES TO SOUTH INDIAN DIET.

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THE supplementary value of various common foodstuffs and certain nutritional factors to poor South Indian diet composed mainly of rice has been the subject of study by several workers. Special mention may be made in this connection of the work of Aykroyd and Krishnan (1937) who have covered a wide field and investigated a number of articles of food from this aspect. They found that, while different foodstuffs showed varying degrees of supplementary effect, the most pronounced results were obtained in the case of calcium (when used as calcium lactate) and dry yeast, both as such and after autoclaving in an alkaline medium. They concluded from these experiments that the chief deficiencies in South Indian diet are calcium and a heat- and alkali-stable factor of the vitamin B-complex, present in yeast. Pal and Singh (1938) studied the supplementary effect of calcium and of phosphorus both singly and in combination with South Indian diet. Their experiments showed that, while phosphorus alone had no supplementary effect on the growth of rats fed on South Indian diet, it slightly augmented the pronounced supplementary effect of calcium when both were used in combination. Swaminathan (1937) determined the biological values of the mixed proteins derived from rice on the one hand and various pulses and skimmed milk powder on the other and from these data he was in a position to get an idea of the supplementary value of the proteins of the latter to rice protein. Skimmed milk powder showed a good supplementary value both by itself and in combination with the common

Indian pulses, but the latter by themselves had a markedly inferior supplementary value.

The modes of utilization of the proteins present in oil-seed cakes, especially ground-nut cake and similar materials, for various purposes, including human consumption, have been the subject of investigation by different workers (Bailey and le Clerc, 1917; Wallis, 1918; Neale, 1926; Payne, 1942; Basu and Sen Gupta, 1946), but no data are available on the supplementary value of the oil-seed cakes, either in raw or processed state, to South Indian diet. This aspect is of special importance in view of the increasing amount of interest in the use of seed cakes as human food. Earlier work carried out at the Nutrition Research Laboratories, Coonoor (Aykroyd and Krishnan, *loc. cit.*; I.R.F.A. Note, 1941) showed that whole ground-nut has practically no supplementary value when added to poor rice South Indian diet. This is rather surprising because ground-nut protein is known to be fairly complete in the nutritional sense (Johns, 1919; Baernstein, 1937; Macheboeuf and Tayeau, 1942). In fact some investigators go to the extent of claiming that the protein of de-fatted ground-nut flour has as high a growth-promoting value as casein itself (Randoin and Boisselot, 1943). Moreover, ground-nut protein has been shown to be a good supplement to the proteins of cereals other than rice, viz. wheat protein (Eddy and Eckman, 1923; Jones and Divine, 1944), corn protein (Maynard, Fronda and Chen, 1923) and oat protein (Smuts and Morais, 1938). It, therefore, promised to be of interest to compare other seed cakes (cotton-seed cake, sesame cake and coco-nut cake) with ground-nut cake in regard to their supplementary values to poor rice South Indian diet and find out whether they also behave in a similar manner.

SUPPLEMENTARY VALUE OF OIL-SEED CAKES TO SOUTH INDIAN DIET.

Supplementary value at the same level of supplement by weight.—In carrying out such a comparative study of the supplementary value of the four seed cakes to South Indian diet, the procedure followed and the details were exactly similar to that described by Aykroyd and Krishnan (*loc. cit.*). Thirty young rats, about 4 weeks' old and weighing nearly 40 g., were divided into five groups, evenly distributed with regard to sex and litter mates. The rats belonging to group I were fed on a basal diet (poor Madrasi diet) the composition of which was similar to that described by Aykroyd and Krishnan (*loc. cit.*). The rats belonging to the other groups were fed on diets composed of the basal diet and 10 per cent supplements of the different seed cakes. The average weekly increase in the body-weights of the rats during a period of six weeks' feeding with the experimental diet is shown in Table II together with the crude protein contents of the diets and the average daily food consumption for each group. Results of analysis for different chemical constituents in the samples of the seed cakes used in the experiment are given in Table I.

The results show that ground-nut cake has the least supplementary value to poor rice South Indian diet, while coco-nut cake, cotton-seed cake and sesame cake have progressively greater supplementary values. It is significant that this is also the order of increasing calcium contents among the four seed cakes (*vide* Table I).

TABLE I.
Analysis of different chemical constituents in oil-seed cakes.

Number.	Description of material.	Moisture, per cent.	Crude protein, per cent ($N \times 6.25$).	Ether extractives, per cent.	Carbohydrates, per cent.	Crude fibre, per cent.	Ash, per cent.	Calcium, per cent.	Phosphorus, per cent.	Iron, mg. per cent.	Vitamin B ₁ , mg. per cent.	Nicotinic acid, mg. per cent.
1	Ground-nut cake	6.63	51.32	8.87	23.72	5.32	4.49	0.073	0.536	2.91	1.06	16.2
2	Cotton-seed cake	7.44	25.08	13.36	32.70	18.75	5.57	0.228	0.437	11.23	0.33	6.7
3	Sesame cake	5.59	33.28	12.23	28.60	8.22	12.08	2.376	0.629	19.30	0.275	5.3
4	Coco-nut cake	11.21	20.87	13.26	39.22	10.51	4.93	0.160	0.489	5.72	0.165	4.1

44 *Supplementary Value of Oil-Seed Cakes to South Indian Diet.*

TABLE II.

Average weekly increase in body-weights of rats fed on experimental diet.

Group number.	Experimental diet.	Quantity of supplement in 100 parts of experimental diet.	Total protein content of the experimental diet, per cent ($N \times 6.25$).	Average daily food intake, g.	Average weekly increase in weight of rats, g.
1	Basal diet (South Indian diet).	...	8.37	7.20	2.8
2	Basal diet and ground-nut cake.	10	13.14	6.95	3.4
3	Basal diet and cotton-seed cake.	10	10.36	7.95	4.9
4	Basal diet and sesame cake.	10	11.08	8.10	5.4
5	Basal diet and coconut cake.	10	9.88	7.90	4.8

Supplementary value at the same level of supplementary protein.—In this case the procedure adopted was the same as before, but while the rats belonging to group I were fed on the basal poor rice diet, the rats belonging to the other groups were fed on diets composed of the basal diet and supplements of the seed cakes in the proportions indicated in Table III. The supplements were added in such amounts that the total protein content of the supplemented diets was maintained at nearly the same level in each case (12 per cent). The average weekly increase in the body-weight of rats belonging to different groups during a period of six weeks' feeding and also the average daily food intake for each group are also given in the same table.

TABLE III.

Supplementary value of supplementary protein.

Group number.	Experimental diet.	Quantity of supplement in 100 parts of experimental diet.	Total protein content of the experimental diet, per cent ($N \times 6.25$).	Average daily food intake, g.	Average weekly increase in weight of rats, g.
1	Basal diet (South Indian diet).	...	8.68	6.95	2.5
2	Basal diet and ground-nut cake.	7.8	12.08	6.80	3.1
3	Basal diet and cotton-seed cake.	20.2	11.97	8.15	6.6
4	Basal diet and sesame cake.	13.4	11.93	8.00	6.1
5	Basal diet and coconut cake.	27.2	12.02	8.30	7.5

The results show that among the oil-seed cakes supplementing South Indian diet, and used at the same protein level, coco-nut cake results in a marked enhancement of growth, while ground-nut cake has a negligible supplementary value. The latter finding is in complete accord with the observations reported from the Coonoor Laboratories (Aykroyd and Krishnan, *loc. cit.*; I.R.F.A. Note, *loc. cit.*). Cotton-seed cake and sesame cake are also found to be quite good supplements to South Indian diet. It is of interest in this connection to note that, while according to Jones and Divine (*loc. cit.*) ground-nut is a fairly good supplement to wheat diet, our experiments and those carried out at the Coonoor Laboratories show that it is not a good supplement to South Indian diet composed mainly of rice.

The values (6.1 to 7.5) obtained for the average weekly increase in weight of rats fed on South Indian diet supplemented with coco-nut, cotton-seed and sesame cakes are comparable to those obtained by Aykroyd and Krishnan (*loc. cit.*) for a 2 per cent supplement of dried yeast (7.2) and somewhat better than for a 7 per cent supplement of casein (4.1).

Owing to their varying protein contents, different percentages of the seed cakes had to be used in the preparation of the diets in order that the protein content might be maintained at the same level. Assuming that the seed-cake protein accounted for about 4 g. in 100 g. of each diet, ground-nut cake diet contained in addition 4 g. of non-protein matter. The corresponding figures for cotton-seed cake diet would be 16 g., for sesame-cake diet over 9 g. and for coco-nut cake diet over 23 g. The contribution of the non-protein matter of the seed cake towards its supplementary value to South Indian diet would thus be relatively important in the experiment described above. This would be particularly so for calcium which is admittedly the limiting deficiency in South Indian diet.

Rôle of fat and calcium in the supplementary value of ground-nut cake.—Preliminary experiments carried out in these laboratories (unpublished work by De, 1946) showed that when the quantity of ground-nut cake added as supplement to South Indian diet was progressively increased, there was a corresponding decrease in the growth rates of young rats fed on these diets. This suggested that though there might not be any definite toxic substance present in ground-nut cake as suggested by Macheboeuf and Tayeau (*loc. cit.*), there might be some growth-inhibiting factor, apart from its low calcium content, which was responsible for the above observation. Mason *et al.* (1945, 1946) found that butter-fat, when added as supplement to South Indian diet, had a marked inhibiting effect on the growth rate of young rats, this effect being counteracted by casein, but not by calcium lactate. This suggested that ground-nut oil present in the seed-cake might be a factor responsible for the observation mentioned above. Such an assumption was not, however, favoured by the experiments of Basu and Nath (1946) who found that inclusion of ground-nut oil in the diet greatly favoured the absorption of calcium and phosphorus and the utilization of these minerals was better than in the absence of the oil. These authors also reported that among the vegetable oils, ground-nut oil has the highest growth-promoting value. Experiments were, therefore, carried out to investigate how far the oil content of ground-nut cake was responsible for its low supplementary value.

Freshly weaned rats, weighing about 40 g. each, were divided into six comparable groups and fed over a period of six weeks with different experimental diets

whose composition is as described in Table IV. In the case of diets fortified with calcium, 1.1 per cent of calcium lactate was incorporated as in the experiments of Aykroyd and Krishnan (*loc. cit.*). The protein contents of the diets and the average weekly increase in the weight of rats are given in the same table.

TABLE IV.

Average increase in weight of rats on experimental diet.

Group number.	Experimental diet.	Quantity of cake supplement in 100 parts of diet.	Quantity of calcium lactate in 100 parts of diet.	Total protein content of experimental diet, per cent ($N \times 6.25$).	Average food intake per rat per day, g.	Average weekly increase in weight of rats, g.
1	Basal diet (South Indian diet).	8.37	6.60	2.6
2	Basal diet and calcium.	...	1.1	8.29	8.85	7.9
3	Basal diet and ground-nut cake.	10	...	11.74	7.15	2.5
4	Basal diet, ground-nut cake and calcium.	10	1.1	11.61	8.50	6.8
5	Basal diet and de-fatted ground-nut cake.	10	...	13.31	6.95	2.7
6	Basal diet, defatted ground-nut cake and calcium.	10	1.1	13.16	7.90	7.7

N.B.—The ground-nut cake used in this experiment was prepared in the laboratory by pressing out oil from fresh ground-nuts and then powdering the meal to pass through a 20-mesh sieve. The oil content was 22.8 per cent. The de-fatted product was obtained by extracting this with ethyl ether in a Soxhlet apparatus.

The results show that there is not much difference between the de-fatted cake and the original sample with regard to their supplementary value, thereby proving that the oil is not the inhibiting factor responsible for the poor supplementary value of ground-nut cake. The value for the average weekly increase in the weights of rats fed on South Indian diet fortified with calcium (7.9) is lower than that obtained by Aykroyd and Krishnan (9). The value for South Indian diet supplemented with de-fatted ground-nut cake and calcium (7.7) was of the same order as for calcium fortified South Indian diet itself (7.9) and somewhat greater than for South Indian diet supplemented with unextracted cake and calcium (6.8).

Supplementary value of seed-cake proteins to South Indian diet protein—biological values of the mixed proteins of the seed cakes and South Indian diet.—In order to investigate merely the value of the proteins of the seed cakes in supplementing the proteins of South Indian diet, experiments were designed to determine the biological (growth-promoting) values of mixtures of proteins derived from South Indian diet on the one hand and the different seed cakes on the other. The biological values were determined by growth experiments on young rats over a period of six weeks' feeding, being computed as average gain in weights of rats per gramme of protein consumed (Osborne and Mendel, 1916; McCollum *et al.*, 1921).

TABLE V.
Composition of the experimental diets.

Number.	Description of diet.	South Indian diet, per cent.	Maize starch, per cent.	Ground-nut cake, per cent.	Cotton-seed cake, per cent.	Sesame cake, per cent.	Coco-nut cake, per cent.	Hydrogenated oil, per cent (m.p. 37° C.).	Salt mixture, per cent.	Calcium lactate, per cent.	Nitrogen, per cent.	Protein, per cent (N×6.25).
1	South Indian diet ...	88	8	3	1	1.149	7.18
2	South Indian diet and ground-nut cake.	44	36.35	7.65	8	3	1	1.152	7.20
3	South Indian diet and cotton-seed cake.	44	28.69	...	15.31	8	3	1	1.117	6.98
4	South Indian diet and sesame cake.	44	32.44	11.56	...	8	3	1	1.158	7.24
5	South Indian diet and coco-nut cake.	44	24.97	19.03	8	3	1	1.225	7.66

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DIET AND HEALTH OF THE SOUTH INDIAN PLANTATION LABOUR.

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DIET SURVEY.

THE diet surveys reported in this paper formed part of an investigation into the family budgets of the plantation labour in India conducted by the Labour Bureau of the Government of India. The region covered by this survey extended over Salem, Coimbatore, Nilgiris and Malabar districts of Madras Province, and Coorg, and included labour on tea, coffee and rubber plantations. Food purchased for consumption over a period of seven days was recorded by the officers of the Labour Bureau on a *pro forma* prepared in consultation with the authors. Since large numbers of observations were involved, actual weighing of foodstuffs was not considered practicable. The total number of persons and adult 'consumption units' distributed among the three types of estates are given in Table I :—

TABLE I.

*The distribution of individuals and 'consumption units'
according to estates.*

Plantation.	Number of estates.	Number of persons.	Total consumption units.
Tea ...	14	1,079	800.4
Coffee ...	17	427	328.0
Rubber ...	4	86	72.8
TOTAL ...	35	1,592	1,201.2

The calorie coefficients used for conversion into 'consumption units' were from Health Bulletin No. 23 (1946). The data were analysed with the use of the tables in the same bulletin. Since complete figures for each estate would occupy too much space, the maximum, minimum and the average consumption of the foodstuffs per consumption unit for all estates are given in Table II:—

TABLE II.

Consumption of foodstuffs by plantation labour.

Foodstuff.	Maximum. g.	Minimum. g.	Average for 35 estates, g.	Number of estates not consuming the particular foodstuff.
Rice ...	556	88	292	...
Wheat ...	267	0	26	27
Millets ...	178	0	21	24
Other cereals ...	323	0	40	26
Pulses and beans ...	185	0	45	4
Vegetables:				
(a) Non-leafy ...	251	6	74	...
(b) Tuberous ...	677	0	73	20
Fruits ...	13	0	1	24
Vegetable oils ...	29	3	12	...
Milk and butter-milk ...	36	0	7	17
Meat and fish ...	94	0	28	1
Sugar and jaggery ...	110	7	41	...
Spices ...	32	8	15	...
Coco-nut, fresh ...	34	0	13	2

Leafy vegetables and ghee were consumed in negligible quantities on all estates and hence have not been included in this table.

The table reveals the fact that the diets were deficient in protective foodstuffs. The consumption of leafy vegetables, milk and eggs was negligible and that of flesh foods extremely meagre. On most estates even the cereal intake was not sufficient. In some cases the deficiency in cereals was being made up by the use of potato, sweet-potato, tapioca or raw plantain. Except in a few instances, the consumption of pulses and beans was low.

The average calorie value of the diets and the average intake of certain nutrients with the range of variation are given in Table III :—

TABLE III.

Intake of calories and certain nutrients by plantation labour.

	Maximum.	Minimum.	Average.
Calories ...	2,903	1,416	2,551
Proteins, g. ...	80	21	49
Fats, g. ...	46	12	22.4
Carbohydrates, g. ...	574	258	408
Calcium, g. ...	1.30	0.10	0.41
Phosphorus, g. ...	1.61	0.52	0.95
Iron, mg. ...	36	7	20
Vitamin A value, I.U.	882	127	460
Thiamine, mg. ...	1.69	0.12	0.67
Ascorbic acid, mg. ...	61	3	19

In Graphs 1 and 2 is illustrated the distribution of consumption units according to the levels of intake of calories, proteins, calcium, phosphorus, iron and ascorbic acid. The standards for comparison used were the recommended daily allowances adopted by the Nutrition Advisory Committee of the Indian Research Fund Association (1944).

Calories.—Nearly 50 per cent of the total consumption units subsisted on less than 2,000 calories per day. In fact, only 2 per cent obtained over 2,900 of food calories and none over 3,000. This fact does show that quantitatively the amount eaten by the labourers fell very much short of the requirements. The observations on the weights of South Indian plantation labour (Table V) proved that 91.5 per cent of the persons examined were underweight by 5 per cent and 79 per cent were underweight by 10 per cent or more below the average weight for the South Indian adults. When one takes into account the low calorie intake among the majority of these labourers, this observation does not seem to be very surprising.

Proteins.—The intake of protein per day was fairly low as compared with the recommended daily allowance of 82 g. The major part of the protein derived from the diet being from rice and hence of a fairly high biological value, cases of protein deficiency were not encountered. An improvement in the protein intake, however, appears to be desirable.

Calcium.—The present observations once again confirm the earlier findings from this Laboratory (Aykroyd and Krishnan, 1937) that poor South Indian diet is particularly deficient in calcium. Nearly 90 per cent of the consumption units could obtain quantities of calcium from their diet which were 40 per cent lower than the recommended daily allowances.

Iron.—The position with regard to iron intake does not appear to be as bad as that with calcium. The prevalence of anæmias among the labourers does not appear to be due to a primary deficiency of iron in the diet but may probably be ascribed to certain other causes which increase the requirements of iron (*vide infra*).

Vitamin A.—The consumption of vitamin A was very low indeed, the highest value computed being 817 I.U. and the lowest 117 I.U. These values are mostly derived from carotene present in vegetable foodstuffs. The highest value is so much less than the level recommended by the Nutrition Advisory Committee that it was not considered worth while to give in Graph 1 the distribution of the levels of consumption. The widespread prevalence of vitamin A deficiency diseases among the plantation labour (*see later*) appears to be the consequence of the extremely low intake of vitamin A and its precursors.

Thiamine.—Figures for the consumption of thiamine also show a low intake. Only about 11 per cent of the total consumption units surveyed obtain over 1 mg. of thiamine or 333 I.U. of vitamin B₁. It was not surprising to find the occurrence of vitamin B₁ deficiency as a major deficiency disease among the plantation labour in South India.

Vitamin C.—The consumption of vitamin C was also below the recommended figure. It was not found possible, however, to demonstrate the prevalence of actual deficiency disease due to lack of vitamin C in the cases examined.

MEDICAL EVALUATION OF NUTRITIONAL STATUS.

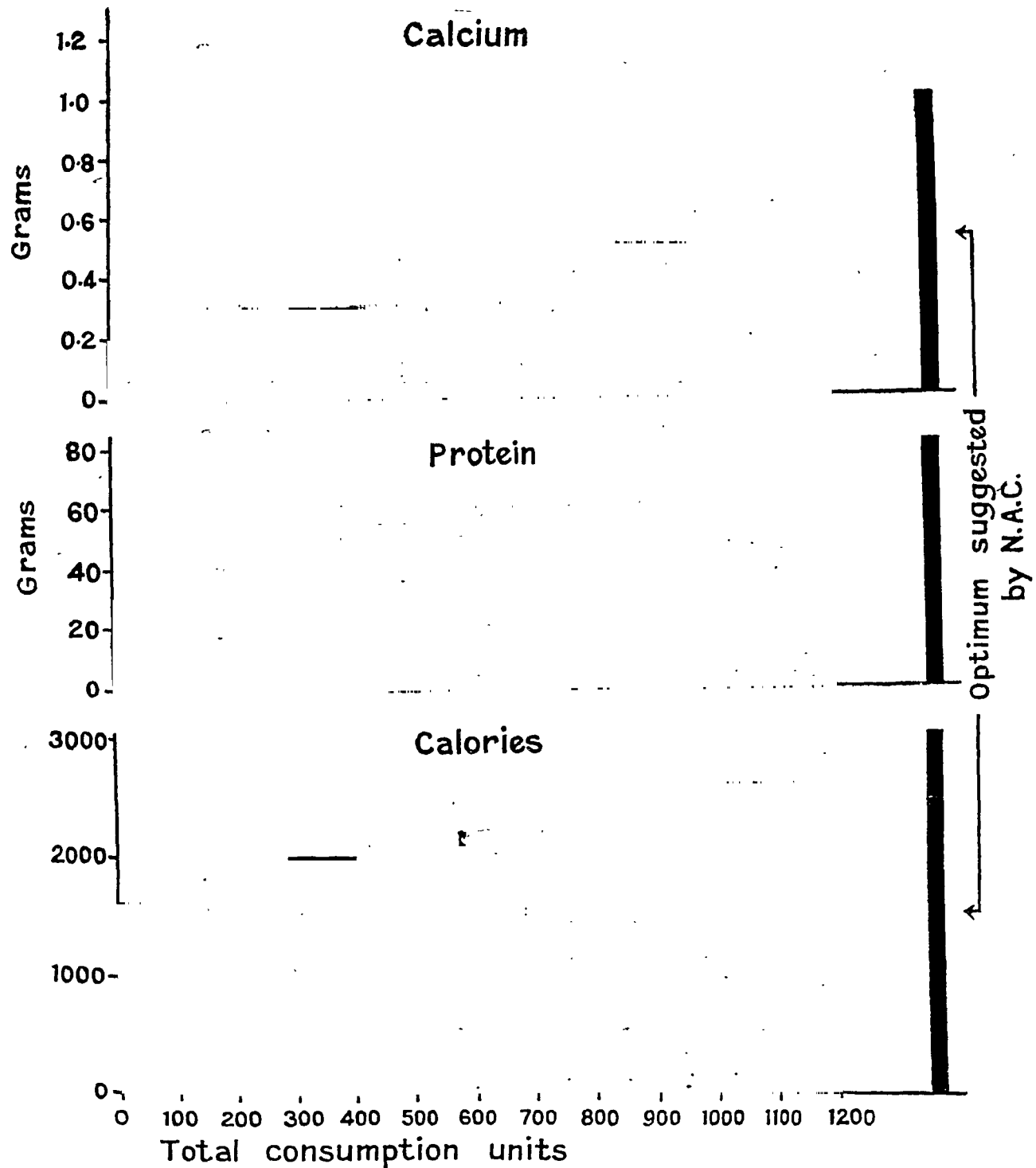
It was not possible to carry out the medical evaluation of nutritional status of all the individuals on each of the estates surveyed. Ten estates were, therefore, selected at random, representing the different plantation regions. The medical survey included 136 families with 461 adults and children of both sexes. Thus, the number examined amounts to 29 per cent of the total number surveyed for diets.

Scope of the examination.

The examination consisted of recording the medical history, measurements of heights and weights, and physical examination. The history was obtained by questioning and with children the information given by them was supplemented by that obtained from the parents.

Physical examination.—The usual routine of a complete physical examination was covered. In addition to the well-established signs of frank malnutrition, special attention was paid to the recognition of mild but chronic nutritional deficiency states. The diseases especially borne in mind were those caused by a deficiency of vitamin A, thiamine, riboflavin, nicotinic acid, ascorbic acid and proteins. Lack of personnel and insufficiency of time precluded more detailed investigations.

GRAPH 1.



The distribution of consumption units according to the levels of intake of calories and certain essential nutrients.
N.A.C.=Nutrition Advisory Committee.

Vitamin B₁

International Units

600

500

400

300

200

100

Vitamin C

Milligrams

60

50

40

30

20

10

0

Iron

Milligrams

30

2

10

0 100 200 300 400 500 600

Total consumption

The distribution of consumption units according to the
N.A.C.=Nutrition Advisory Committee.

For the detection of vitamin A deficiency, reliance was mainly placed on a detailed inspection of the morphological changes in the conjunctiva and skin. In spite of the present controversial stage of the subject, follicular hyperkeratosis, ranging from a few parakeratotic follicles to extensive eruption was also taken as indicative of vitamin A deficiency. While Bitot's spots were included under advanced deficiency, contemporary controversy about the value of localized elevation, thickening and pigmentation of the conjunctiva as signs of early vitamin A deficiency has been taken into account (Nicholls and Nimalasuriya, 1939; Kruse, 1941; Kodicek and Yudkin, 1942; Passmore, 1947).

For thiamine deficiency, the authors had to rely exclusively on the subjective and objective evidence of peripheral neuropathy. Subjective sensory disturbances have been recorded. Particular attention was paid to the peripheral nervous system in the neurological examination. The state of the knee and ankle jerks together with the presence or absence of calf-muscle tenderness and plantar hyperæsthesia were recorded. It must, however, be emphasized that these signs are not definitely pathognomonic of thiamine or any other nutritional deficiency.

Seborrhœic changes in the face, fissuring at the angles of the mouth and lips, and magenta tongue were taken to indicate riboflavin deficiency.

For detection of nicotinic-acid deficiency, the well-known changes in the skin, mouth and tongue were looked for. Vitamin C inadequacy was judged by the characteristic changes in the skin and gums, and protein deficiency by dependent œdema, not attributable to cardio-renal causes.

Hæmoglobin determination.—The determination was carried out on all subjects with the Dare's hæmoglobinometer. Although the instrument had not been actually calibrated, the results were found to compare favourably with those obtained with the photo-electric colorimeter. The same person carried out the estimations throughout the investigation.

In Tables IV to IX are given the relevant findings of the investigation :—

TABLE IV.
Age and sex distribution of subjects.

Age range.	NUMBER OF PERSONS.		
	Male.	Female.	Total.
Under 12 years (children) ...	75	88	163
12 to 19 years ...	17	24	41
20 to 29 years ...	34	60	94
30 to 39 years ...	72	54	126
40 and over ...	31	6	37
TOTAL ...	229	232	461

Heights and weights.—The data on height and weight of adults were compared with the charts of the Oriental Life Assurance Company for Madras Hindus and Muslims. In Table V are given the data for 220 adults between the ages of 20 and 50 years. As comparable information was not available for children, no attempt to classify the observations has been made.

TABLE V.

Deviation of weights from the average for South Indian adults.

Deviation from average.	Number of persons.	Per cent of total number of men.
20 per cent or more above average ...	Nil	Nil
10 to 20 per cent above average ...	1	0.45
5 to 10 per cent above average ...	2	0.9
0 to 5 per cent above or below average ...	16	7.27
5 to 10 per cent below average ...	26	11.81
10 to 20 per cent below average ...	94	42.73
20 per cent or more below average ...	81	36.81
TOTAL ...	220	...

Vitamin A deficiency.—In Table VI are tabulated the observations pertinent to vitamin A deficiency among the subjects:—

TABLE VI.

Vitamin A deficiency in adults and children.

Physical findings.	CHILDREN UNDER 12 YEARS.		CHILDREN OVER 12 YEARS AND ADULTS.	
	Number of cases.	Per cent of total.	Number of cases.	Per cent of total.
Definite evidence of vitamin A deficiency (either dermal or ocular).	41	25.6	39	13
Follicular hyperkeratosis ...	39	23.6	23	7.7
Bitot's spots ...	12	7.3	23	7.7
Localized elevation, thickening, pigmentation and opacity of the scleral conjunctiva.	100	61.3	242	81.2
TOTAL ...	163	...	298	...

Of children 25·6 per cent and of persons above 12 years 13 per cent showed advanced states of vitamin A deficiency as judged by gross inspection of the morphological changes produced in the conjunctiva and skin. On the other hand, 81·2 per cent of persons above 12 years and 61·3 per cent of children showed evidence of conjunctival degeneration, such as elevation, thickening, etc., which, according to Kruse (*loc. cit.*), are to be rated as evidence of early deficiency of vitamin A.

Thiamine deficiency.—No cases of cardiac beri-beri were encountered. The findings of the neurological examination are set forth in Table VII:—

TABLE VII.

Evidence of thiamine deficiency in 298 persons above the age of 12 years.

Physical findings.	Number of cases.	Per cent of total.
Calf-muscle tenderness	84	28·2
Plantar hyperæsthesia	45	15·1
Absence of ankle jerks	33	11·1
Number of persons with one or more of the above signs.	92	30·8
Subjective sensory disturbances complained of such as tingling and numbness, pins and needles, etc.	102	34·2

Of the subjects 30·8 per cent showed evidence of involvement of nervous system, males being more frequently involved than females. There were only 2 cases of peripheral neuropathy seen in children.

Riboflavin deficiency.—Table VIII summarizes the incidence of signs of riboflavin deficiency. Of the 461 persons examined including children, 12·1 per cent showed one or more signs of this deficiency.

TABLE VIII.

Incidence of riboflavin deficiency.

Age range.	Total number examined.	Number showing one or more signs of deficiency.	Per cent of total.
Persons over 12 years ...	298	39	13·2
12 years and below ...	163	18	11·0

Other deficiencies.—There were no cases of pellagra. Two children showed spongy hypertrophic gums, easily bleeding on touch, not associated with hæmorrhagic manifestations anywhere else. There were no cases of œdema deficiency.

Hæmoglobin.—Table IX contains a summary of the hæmoglobin findings. Taking the values from 13·2 g. to 16·5 g. per cent as the range of normalcy, 291 persons showed subnormal hæmoglobin levels; out of these, 148 had less than 9·8 g. per cent of hæmoglobin. Stool examination of all these cases was not done. In one locality (Coorg) where it was done, 24 out of the 52 cases examined showed the presence of hookworm ova. Thus, there is evidence to suggest that many cases of low hæmoglobin may presumably be due to ankylostomiasis.

TABLE IX.

Hæmoglobin levels in 338 persons including children.

Range of hæmoglobin level g. per 100 c.c.	NUMBER OF PERSONS.	
	12 years and under.	Above 12 years.
16·5—14·9	2	22
14·8—13·2	3	20
13·1—11·5	7	54
11·4—9·9	15	67
9·8—8·2	46	57
8·1—6·6	11	17
6·5—4·9	1	7
4·8—3·3	8
Less than 3·3	1
TOTAL	85	253

Other illnesses.—Intercurrent illnesses play an important rôle in producing secondary deficiencies. Malaria as assessed by palpable spleen was not detected in most of the estates. Only two estates betrayed its presence, one of them being heavily infected. The splenic index in Coorg was as high as 50 per cent, whereas in Thiruvambadi (Malabar) it was 11·1 per cent. Skin infections, such as scabies and coccal dermatitis, were frequently encountered though exact figures cannot be given. Upper respiratory tract infections were common, chronic lung disease

however was rarely seen. Two cases of acute lobar pneumonia were encountered during the examination in one estate. Only one case of rheumatic heart disease was met with. Pathological features referable to the cardio-vascular system consisted of those found in advanced anæmia, such as dilated hyper-dynamic heart with systolic murmur. There were no cases of cirrhosis of the liver nor of infective hepatitis. Histories suggestive of chronic gastro-duodenal ulceration were obtained occasionally. Diarrhœa was frequently complained of, more especially among children. Over half of them were with blood and mucus.

Attention must be drawn to some features in the medical histories which are not included above, features which are mainly referable to gastro-intestinal dysfunction, such as distension, lack of appetite, indigestion, etc. There are indications in the literature (Bean and Spies, 1940) that gastro-intestinal discomfort can frequently be relieved by the administration of one or more components of vitamin B complex. It is possible, therefore, that some at least of these complaints may have been the result of malnutrition.

SUMMARY.

1. The diets of labourers and their families on 35 tea, coffee and rubber plantations in South India were surveyed. The number of individuals included in the survey was 1,592.

2. The survey revealed that the diets were low in calories and proteins and grossly deficient in calcium, vitamin A and its precursor and thiamine.

3. The nutritional status of 461 individuals from 10 estates selected at random was medically evaluated.

4. The nutritional survey showed that—

- (a) Seventy-nine per cent adults were underweight by 10 per cent or more as compared with the average for South Indian adults.
- (b) Of individuals above 12 years 81·2 per cent and of children below 12 years 61·3 per cent showed morphological changes attributable to early vitamin A deficiency.
- (c) Evidence of neurological involvement due to thiamine deficiency was found in 30·8 per cent individuals above 12 years of age.
- (d) Of the total number examined 12·1 per cent showed one or more signs of riboflavin deficiency.
- (e) Two hundred and ninety-one persons had hæmoglobin levels below 13·2 g. and 148 had less than 9·8 g. per cent. Some evidence is forthcoming to suggest that the cause of anæmia may lie in extensive incidence of ankylostomiasis.

The authors are indebted to the officers and staff of the Labour Bureau, Government of India, led by Mr. R. V. Mathai, M.A., M.Litt., for collecting data for dietary intake. They are also grateful to the Ministry of Labour, Government of India, for permission to publish the results of diet and nutrition surveys.

Much interest has recently been created by the works of Shaw Dunn, McLetchie and Sheehan (1943) who have shown that both hyperglycaemia and characteristic damage of the insulin-secreting cells of the pancreas can be brought about by the continued administration of Alloxan. The β -cell degeneration, however, caused by Alloxan seems to be different from that observed by Best *et al.* (1943) resulting from the continued injection of anterior-pituitary extract.

In a recent publication the authors have shown (Nath and Brahmachari, 1944) that aceto-acetic ester and the sodium salts of β -hydroxy butyric acid and pyruvic acid when injected into rabbits can produce an immediate rise in the blood-sugar level, which comes down gradually to the normal limit after about 24 hours. Subsequently, in another publication they have shown (Nath and Brahmachari, 1946) that the action of insulin is inhibited both *in vitro* as well as *in vivo* by these toxic metabolites.

An investigation was, therefore, begun with a view to study the direct relationship between these intermediary fat metabolites and hyperglycaemia and to see whether these factors may be held responsible for the characteristic damage to the islet cells of the pancreas in the long run, as observed so commonly in case of the 'diabetes mellitus'.

The present paper deals with the effect of continued daily administration of some intermediary fat metabolites on the blood-sugar level of rabbits.

EXPERIMENTAL.

The substances studied were (i) aceto-acetic acid and (ii) β -hydroxy butyric acid, in the form of their sodium salts. A set of healthy male rabbits weighing about 2 kg. and having a fasting blood-sugar value between 110 mg. and 120 mg. per 100 c.c. were selected for these observations. These rabbits were divided into two groups, one of those substances being injected daily to each group continually for a long period. The rabbits were placed in wooden cages, two of them being placed in each cage. During the whole experimental period, they were given green gram and green grass as food. Blood was taken from the marginal veins of the ears of the animals and the blood sugar was estimated by the method of Hagedorn and Jensen (1923) from the samples collected after the animals were kept in a fasting condition for about 18 hours.

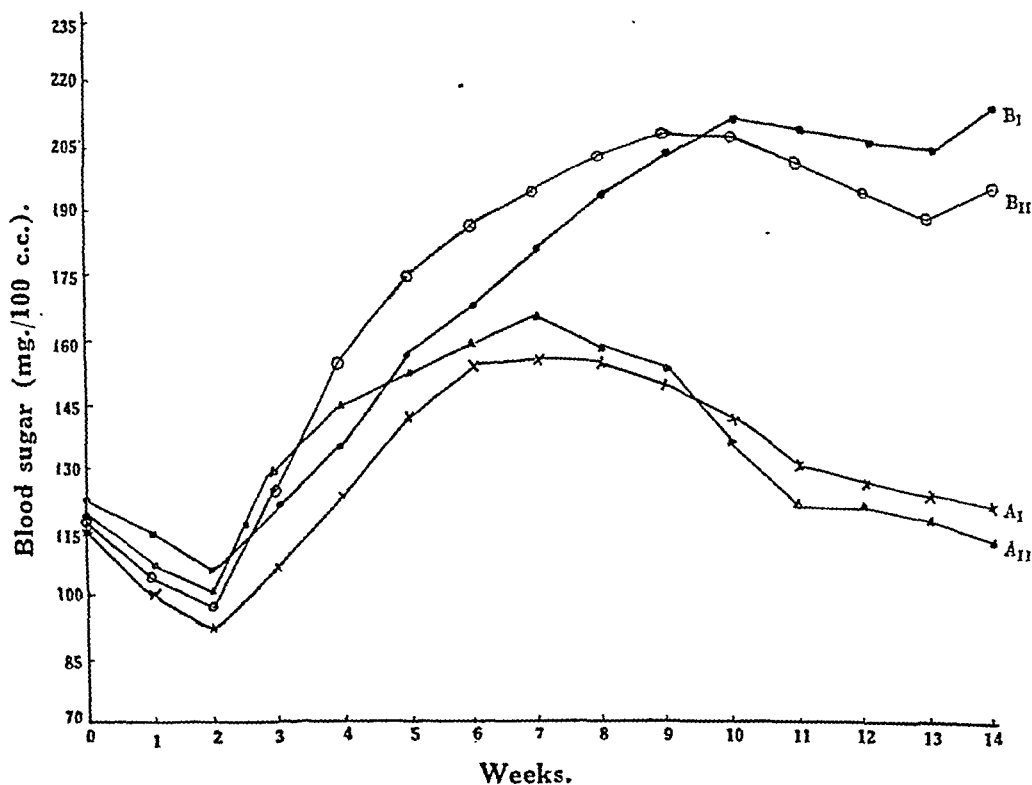
In the first set of animals receiving a constant dose of 50 mg. of substance per day it was seen that the hyperglycaemia caused by these substances did not persist for a long time, unless the doses of the substances injected were also gradually increased. So, in a second series of experiments, the animals were given gradually increasing doses of the substances beginning with a daily dose of 50 mg. per animal which was increased weekly by 15 mg. The total period of observation was 14 weeks.

In a third set of animals (2 rabbits) 100 mg. of β -hydroxy butyric acid were injected daily and the dose was increased by 100 mg. more only after distinct signs of hypoglycaemic tendency had been observed.

In a fourth set of experiments, in which four animals were given gradually increasing doses of β -hydroxy butyrate, the effect on the 'sugar tolerance' was observed at some regular intervals throughout the whole experimental period. Observations on the fasting levels of the blood sugar were made every seven days, blood for examination being withdrawn before the usual daily injection. The rabbits were weighed every week, and the general health of the rabbits was examined every day before giving injections. The urine was examined for sugar and acetone bodies once a week.

Injections were given intramuscularly every day after food. Strict antiseptic measures were followed during the process of injections so that in no case was there any untoward effect, such as inflammation or wounds, etc., due to these long-continued daily injections.

GRAPH 1.



A_I → Constant aceto-acetate doses.

B_I → Increasing aceto-acetate doses.

A_{II} → Constant β -hydroxy butyrate doses.

B_{II} → Increasing β -hydroxy butyrate doses.

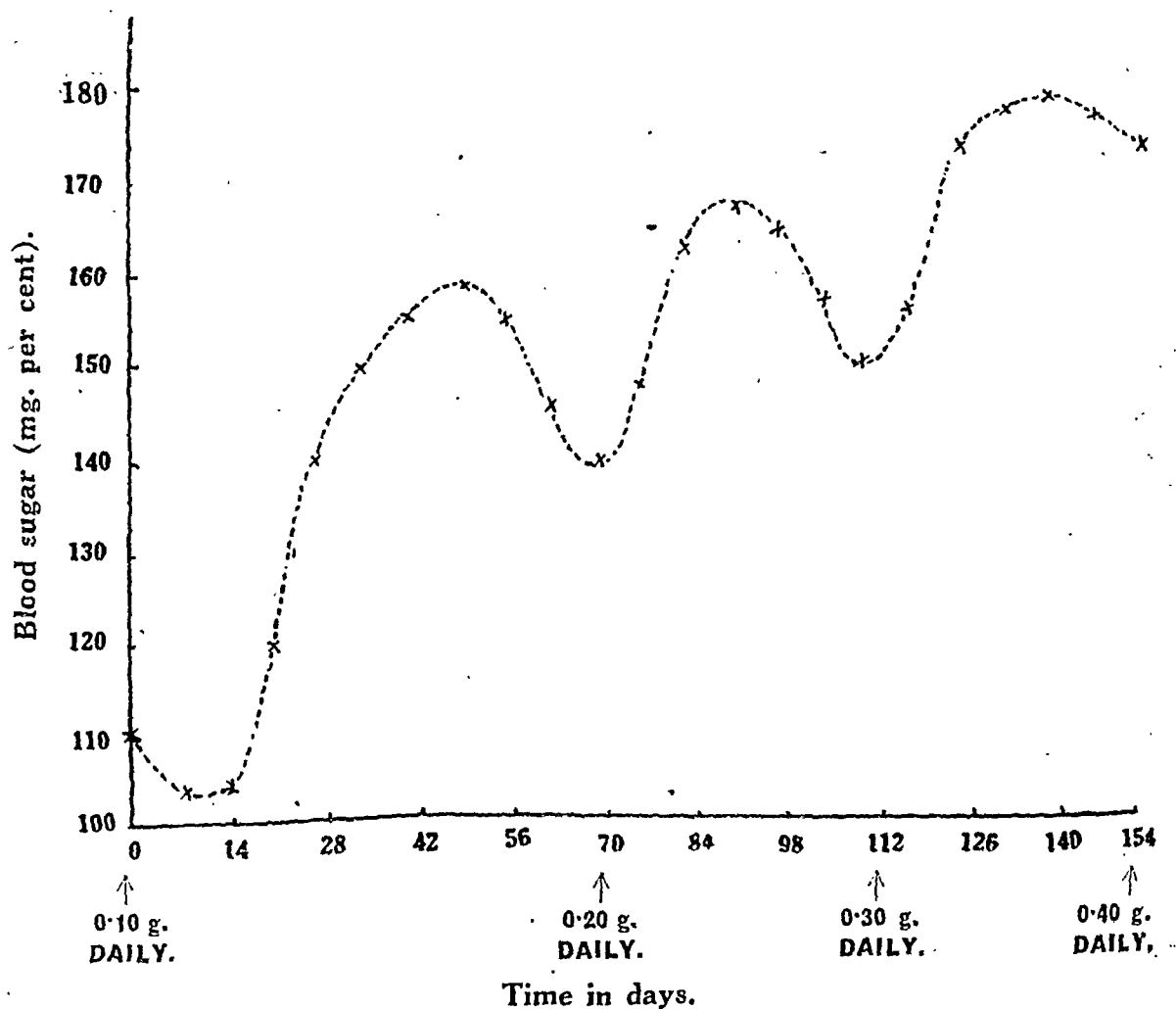
RESULTS.

Gradual changes in the blood-sugar values are given in Table I, and the results with only one from each group of animals are represented in Graph 1 in order to

indicate the nature of their initial fall and gradual rise after a particular period. Table II shows the results of observations on the body-weights of these animals receiving gradually increasing doses of the substances; Graph 2 shows the blood-

GRAPH 2.

Effect of 'increasing doses'.

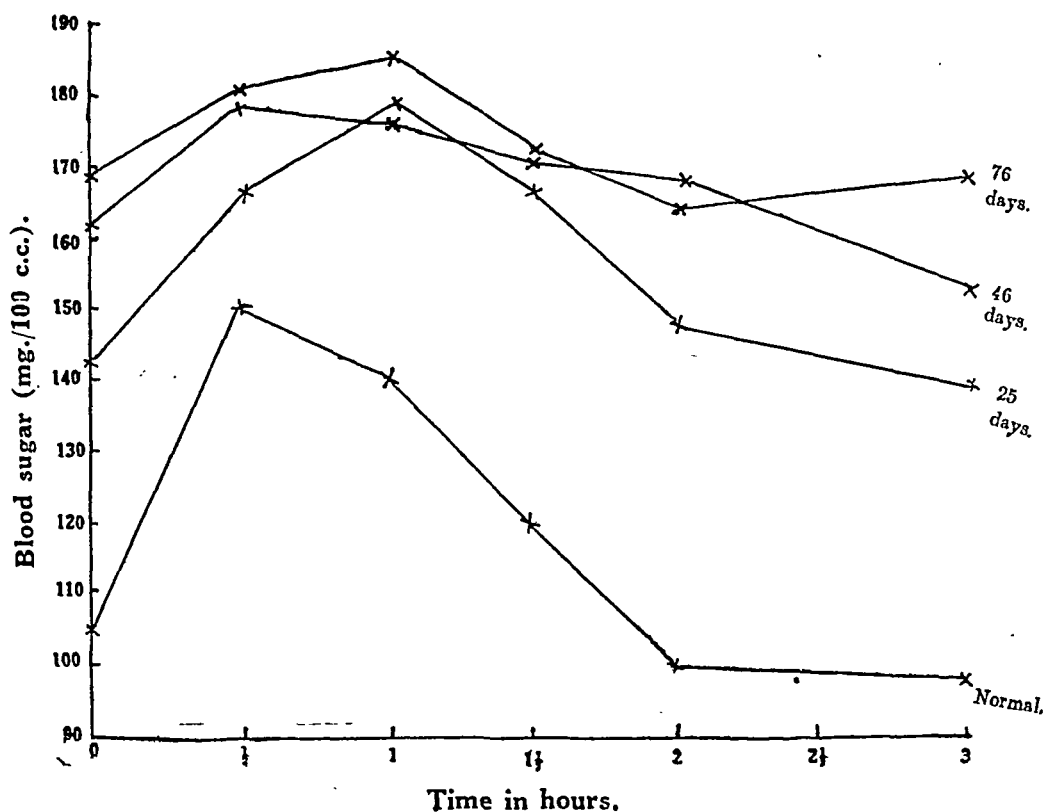


sugar results after increasing the dose only when a marked fall in the blood-sugar values were observed; while Graph 3 represents the sugar-tolerance curves at different stages. Results showing the development of glycosuria in the animals are represented in Table III.

Some observations with the female animals.—Two of the experimental animals receiving sodium aceto-acetate happened to be female and they could not stand injection for more than 4 weeks. The values of blood sugar in these animals were

GRAPH 3.

Sugar-tolerance curves.



found to rise from 117 mg. and 126 mg. to 168 mg. and 159 mg. respectively as studied on the 21st day. This observation confirmed the findings of Deuel and Davis (1942) who noted that the female experimental animals are more prone to ketosis than the males. Both these animals died during the end of

TABLE I.

Fasting blood-sugar values (mg./100 c.c. blood).
(Average values for two rabbits in each cage.)

(A) CONSTANT DOSES (50 MG. PER ANIMAL PER DAY).																	
Cage No.	Rabbit No.	Solution injected.	Blood-sugar values after time intervals in weeks:														
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
I	7 and 8	Aceto-acetate 50 mg. per day per animal.	110	100	85	73	88	120	146	155	166	160	153	136	138	130	126
II	9 and 10	"	120	100	93	115	130	149	159	154	156	149	130	124	118	116	118
III	11 and 12	"	116	100	95	130	149	154	158	156	140	145	138	129	120	124	118
IV	13 and 14	β -hydroxy butyrate 50 mg. per day per animal.	118	116	100	130	149	158	160	165	158	145	137	120	124	115	112
V	15 and 16	"	120	100	100	126	136	149	159	168	160	158	138	120	129	130	110
VI	17 and 18	"	115	110	100	128	148	151	156	165	158	150	136	124	125	120	115
(B) GRADUALLY INCREASING DOSES (DOSE REPRESENTS MG. PER ANIMAL PER DAY).																	
Dose (mg.) :—			50	65	80	95	110	125	140	155	170	185	200	215	230	245	260
VII	20 and 21	Aceto-acetate	116	110	98	125	139	160	170	184	200	210	215	210	212	200	218
VIII	22 and 23	"	120	116	106	118	128	150	160	174	190	200	210	204	208	212	214
IX	24 and 25	"	118	116	112	123	137	160	175	183	192	200	205	215	198	200	210
X	26 and 27	β -hydroxy butyrate.	120	112	110	128	156	172	185	192	205	210	214	218	200	198	196
XI	28 and 29	"	112	90	95	126	160	185	198	210	216	220	218	200	195	190	200
XII	30 and 31	"	118	110	100	125	150	165	175	180	185	193	190	185	192	180	186

TABLE II.

Average body-weight of the experimental rabbits receiving gradually increasing doses of (i) aceto-acetate and (ii) β -hydroxy butyrate.

(Average values for six rabbits in each group.)

Group.	Average values for rabbit No.	Record of body-weight (g.) with time in days :											
		Original.	7	14	21	28	35	42	49	63	77	84	98
I													
Aceto-acetate ...	20, 21, 22, 23, 24 and 25.	1,986	1,888	1,980	1,989	1,964	1,960	1,950	1,942	1,800	1,730	1,705	1,668
II													
β -hydroxy butyrate	26, 27, 28, 29, 30 and 31.	2,008	2,000	2,020	2,001	1,980	1,964	1,924	1,868	1,800	1,764	1,740	1,704

TABLE III.

*Test for glycosuria in the experimental animals.*Substance injected, β -hydroxy butyrate (intramuscularly).

(Initial dose 50 mg. daily per animal, increased by 15 mg. per week per animal.)

(Average figures for 4 rabbits.)

Period of injection, days.	Total amount injected from beginning.	Blood sugar, mg./100 c.c.	TEST FOR GLYCOSURIA:		
			Fasting.	After food.	After feeding glucose (5 g.).
7	350 mg.	132
14	805 mg.	149
21	1·365 g.	158
28	2·030 g.	159	+ (trace)
35	3·800 g.	163	+ (trace)
42	4·675 g.	165	+ (trace)
49	5·655 g.	162	...	+ (trace)	++ (0·84%)
56	6·740 g.	168	+ (trace)	++ (0·5%)	+++ (1·33%)
63	7·930 g.	173	++ (0·08%)	++ (0·9%)	+++ (1·8%)
70	9·225 g.	170	++ (0·08%)	++ (0·93%)	...
77	10·625 g.	168	++ (0·06%)	++ (0·8%)	+++ (1·5%)
84	12·130 g.	172	++ (0·06%)	++ (0·84%)	+++ (1·2%)

4th week and on autopsy the following changes in their glands and organs were observed :—

Animals Nos. I and II (F):

(a) Cyanosis of face, (b) looseness of hair, (c) rupture of the lungs, (d) blackening of the liver and kidneys, (e) damage to the pancreas and (f) peritonitis.

Details regarding the histological findings of the essential glands of the experimental animals, which are in progress, will be sent for publication in due course.

DISCUSSION OF RESULTS.

It will be seen from Graph 1 that, for the first few days after the beginning of the daily injections (dose 50 mg.), the general tendency of blood sugar is towards a

fall, thus showing rather a stimulus to the insulin-secreting mechanism of the pancreas. This has been followed in every case by a gradual rise, the maximum value being attained within 49 to 56 days. After this period the blood sugar remains practically constant for some time, provided the strength of solutions injected remains the same, and then there is a tendency towards a gradual fall. At the end of about 90 days of injection, the blood sugar actually fell to the pre-experimental level, and in some cases even below that. This tendency towards hypoglycaemia persists, if the strength of solutions injected is not increased gradually. Graph 2 shows how hyperglycaemia can be maintained by gradually increasing the dose of the injectule whenever there is a tendency for the blood-sugar level to fall.

It is also interesting to note that, whereas the animals receiving constant doses initially lost some weight and then maintained more or less constant body-weight, those of the second group receiving gradually increasing doses went on losing weight and at a later stage became very much emaciated before death.

Graph 3 shows the change of sugar tolerance of the animals receiving gradually increasing doses of β -hydroxy butyrate. At the last stage of the experiment the curves are found similar to those of the typical serious diabetic types.

SUMMARY.

1. Sodium salts of the intermediary fat-metabolism products, viz. aceto-acetic acid, β -hydroxy butyric acid, while injected daily in the normal rabbits, cause initial reduction of blood sugar up to a period of 2 weeks or so.

2. After this period of 2 weeks' time there is observed a gradual rise in the value of blood sugar which after 3 or 4 weeks exceeds the normal limit in almost all the cases.

3. This state of hyperglycaemic condition is not maintained for long after a particular time, and the blood-sugar values begin to fall, if the daily dose is not allowed to be increased.

4. By gradual increase in the doses of these compounds it has been possible to maintain a typical type of hyperglycaemia characterized with decreased sugar tolerance, as in the case of clinical diabetes mellitus.

5. Glycosuria and acetonuria have been observed along with hyperglycaemia in the animals (rabbits) receiving daily injection of gradually increasing doses of β -hydroxy butyrate for about 50 days.

6. The results confirm the hypothesis put forward by the authors (Nath and Brahmachari, 1944; 1946) that the ketone bodies might first stimulate the pancreatic islet cells and later cause some disturbances in the activity of those cells after fatigue through excessive work.

The authors are grateful to Shrimant M. G. Chitnavis for his donation for this Department and to the Lady Tata Memorial Trust for the award of a scholarship to one of them (H. D. B.).

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STUDIES ON THE ÆTIOLOGICAL FACTORS RESPONSIBLE FOR THE ONSET OF DIABETIC SYMPTOMS.

Part II.

EFFECT OF LONG-CONTINUED ADMINISTRATION OF SOME FAT-METABOLISM PRODUCTS ON THE POTENCY OF PANCREATIC INSULIN OF GUINEA-PIGS.

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JOSLIN (1933) and many others regard adiposity as a possible precursor of diabetes. Roy and Mukherjee (1939) have shown that the first stage in the development of hyperglycæmia is a condition of acidosis, resulting from the faulty method of living especially through high-protein and fat diet. Nath and Brahmachari (1944), who for the first time began some systematic investigations on the rôle of the intermediary fat metabolites on the development of diabetic conditions in animals, showed that gradual accumulation of those products (e.g. β -hydroxybutyric acid, aceto-acetic acid, etc., in the form of their sodium salts) can cause a state of hyperglycæmia in the experimental animals (rabbits).

Subsequently, the authors (Nath and Brahmachari, 1946) have also shown that these substances have the power of inhibiting the activity of external insulin both *in vitro* and *in vivo*.

The present observation deals with the effect of these ketone bodies on the potency of pancreatic insulin of the guinea-pigs.

* Lady Tata Memorial Scholar.

EXPERIMENTAL.

The experiments were performed on adult male guinea-pigs, all having almost the same body-weight, i.e. about 500 g. A set of twelve male guinea-pigs was selected for each set of experiments, thus requiring three dozen guinea-pigs for three sets. Besides these, twelve guinea-pigs were used for control experiments.

The experimental animals were given daily injections of the sodium salts of the abnormal fat metabolites, such as aceto-acetic acid, β -hydroxy-butyric acid and pyruvic acid, each set of animals receiving one substance continuously. The initial dose in each case was 100 mg. per animal and this was increased by 15 mg. per animal per week. All the animals were kept on their usual diet consisting of green gram and green grass.

Three animals from each set were de-pancreatized at a time, at regular intervals, and the insulin extracted from the pancreas was assayed on normal healthy rabbits. The results were evaluated as insulin activity per g. of the pancreas-tissue. Insulin was extracted from the pancreatic tissue according to the method of Best *et al.* (1939) and the assaying was done according to Marks (1936).

RESULTS.

The percentage reduction of blood sugar of normal healthy rabbits caused by the insulin obtained per gramme of pancreatic tissue of the animals is given in the Table and the gradual changes in the potency of the pancreatic insulin are represented in the Graph.

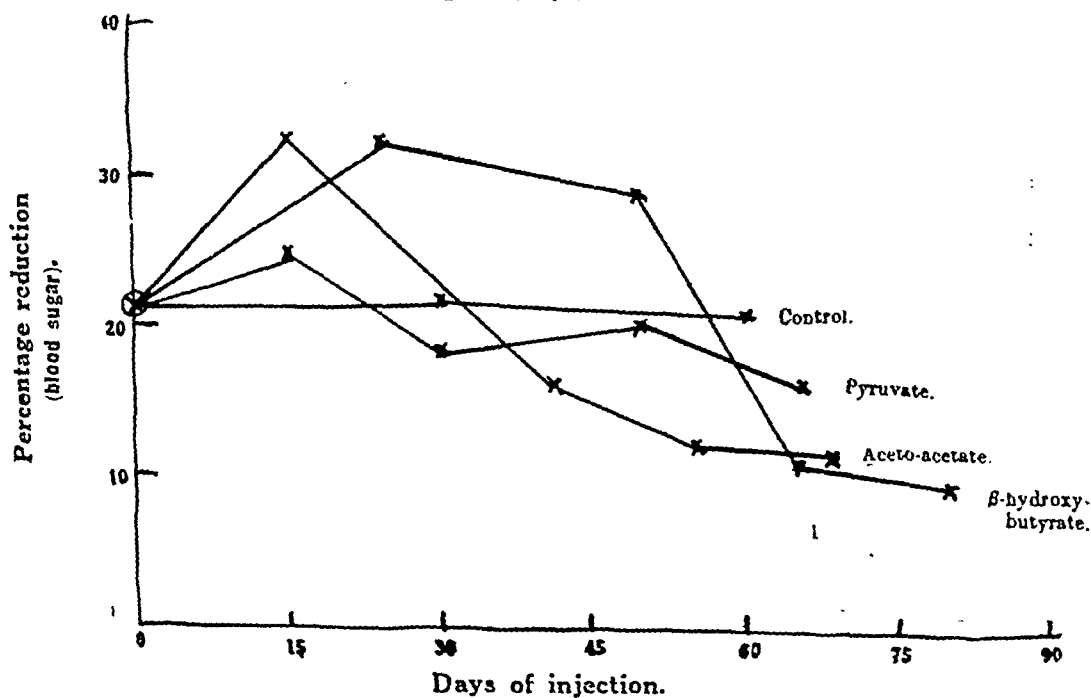
TABLE.

Substance studied.	Days of injection before de-pancreatization.	ASSAY OF INSULIN OBTAINED, PER G. OF PANCREAS-TISSUE (ON NORMAL MALE RABBITS).		
		Original blood sugar, mg.	Average blood sugar of 5 hours' pool, mg.	Per cent reduction.
1. Aceto-acetate ...	15	100	67	33.0
	41	104	87	16.3
	56	98	86	12.4
	68	110	97	11.8
2. β -hydroxy-butyrate ...	24	104	70	32.6
	50	110	78	29.0
	65	96	85	11.4
	80	95	86	9.5

TABLE—concl'd.

Substance studied.	Days of injection before de-pancreatization.	ASSAY OF INSULIN OBTAINED, PER G. OF PANCREAS-TISSUE (ON NORMAL MALE RABBITS).		
		Original blood sugar, mg.	Average blood sugar of 5 hours' pool, mg.	Per cent reduction.
3. Pyruvate ...	15	102	76	25.0
	30	108	87	18.3
	50	100	80	20.0
	65	105	88	16.2
Normal (control) ...	0	105	82	21.9
	30	100	78	22.0
	60	100	79	21.0

GRAPH.

Changes in 'potency of pancreatic insulin'.

DISCUSSION.

From a study of the results it is evident that injection of the intermediary fat metabolites causes an initial increase in the activity of insulin per g. of the pancreatic tissues of the treated animals. Such gradual increase in potency was observed only up to 2 to 3 weeks of injection there being a sudden fall, most pronounced in case of animals receiving aceto-acetate, thereafter.

The pancreatic activity, as far as the potency of insulin is concerned, is found to come below the normal level within 4 weeks' time in case of animals treated with aceto-acetate, while those treated with β -hydroxy-butyrate show depression below normal limits after about a period of 7 weeks; and insulin potency of the animals of both the groups is found to be reduced by more than 50 per cent after about 2 months' time.

This type of initial increase in the blood-sugar reducing capacity of the pancreatic extract and a sudden decrease after a short period of treatment with different types of intermediary fat metabolites confirms very nicely the postulation made by the authors a few years ago (Nath and Brahmachari, 1944) where it was suggested that these products might first stimulate the pancreatic islet cells which might gradually become fatigued through excessive work.

The initial hypoglycæmic effect of these types of compounds for a period of 2 to 4 weeks and their late effect of causing hyperglycæmia with increased doses, as recorded in Part I (Nath and Brahmachari, 1949), can also be accounted for very nicely through this concept.

The data also show well that these ketone bodies, when injected into the normal animals for a long period, exert their hyperglycæmic effect not through any stimulus to the adrenals but through their activity in the proper insulin-secreting mechanism of the pancreas.

It can be noted, however, that the pyruvate does not seem to have exerted such great effect as observed with the substances mentioned previously. This might be due to its less toxic nature, being a normal intermediary metabolite of the carbohydrates as well.

This is well known that during some disturbance in the proper metabolism of fat in the body the concentration of these types of ketone bodies increases to a considerable extent and obese persons are more likely to have such disturbances in their systems. The present observation can, therefore, throw some light into the matter how obesity and overweight are, in most cases, found to lead to the development of diabetes in the long run.

SUMMARY.

1. Intermediary fat metabolites, such as β -hydroxy-butyric acid and acetoacetic acid (Na salts), when injected into normal guinea-pigs beginning with the daily dose of 100 mg. per animal, have been found to increase the blood-sugar reducing capacity of the pancreas of those treated animals initially, i.e. up to 2 to 4 weeks' time, thus indicating initial stimulus to the insulin-secreting mechanism of that gland.

2. Continued injection of these substances, however, for a longer period brings about gradual reduction in the insulin activity of the pancreas ultimately leading to only one half of its potency after a period of two months, which indicates ultimate fatigue of the islet cells caused through constant stimulation and strain.

3. Pyruvate does not seem to be exerting such great influence in the disturbance of the secretion of insulin as the other intermediary fat metabolites.

4. Some insight into the matter as to how adiposity, in most cases, acts as a precursor to diabetes, which generally develops after a particular period of life, has been obtained.

Our grateful thanks are due to Shrimant M. G. Chitnavis for his kind donation for the development of this Department and active interest in our research works.

We also record our thanks to the Lady Tata Memorial Trust for encouraging this piece of investigation by the award of a scholarship to one of us (H. D. B.).

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INFANT FEEDING EXPERIMENTS WITH SOYA-BEAN MILK.

BY

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An Inquiry under the auspices of the Indian Research Fund Association.

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PREVIOUS studies carried out by us on the nutritive value of soya-bean milk by feeding on rats and adult human subjects have shown that both the milk and the curd prepared from it are easily digested and absorbed by the body. The actual biological value as determined by metabolism experiments was found to be about 90 per cent as good as that of cow's milk fed under similar conditions (Desikachar *et al.*, 1946). Similar observations were extended on children to study the suitability of soya milk for infants and toddlers. The results of such feeding experiments on children are embodied in this communication.

It has been reported by earlier workers that the fat content of Indian cow or buffalo milk is fairly high specially in the winter months (Rangappa, 1948). Owing to this high fat content, it is a general experience that such milks are often not easily digested by children especially in South India. Soya-bean milk on the other hand contains a relatively lower fat content and on that account might prove useful as an infant food. Moreover, soya-bean milk is being fed to infants in China (Chang and Ernest, 1931) and sweetened soya-bean curd is also reported to be beneficial as an infant food because its curd tension is low. It was on these

TABLE—*contd.**Calcium balance.*

(All data represent figures in mg. for the total period.)

Subject.	Age, years.	Intake in g.	Urinary excretion in g.	Fæcal excretion in g.	Total excretion in g.	Amount retained in g.	Percentage retained.
SOYA MILK :							
L. M. ...	2	4,055	181	1,785	1,966	2,089	51·52
A. C. ...	2	3,898	146	1,732	1,878	2,020	51·82
S. M. ...	2	4,434	128	1,686	1,814	2,620	59·09
P. F. ...	3	4,490	109	2,143	2,252	2,238	49·83
H. S. ...	2	4,486	98	2,341	2,439	2,047	45·63
N. S. ...	1	4,484	107	1,947	2,054	2,430	54·20
Average	52·4
COW'S MILK :							
L. M. ...	2	5,868	175	2,759	2,934	2,934	50·00
A. C. ...	2	5,868	178	2,711	2,889	2,979	50·76
S. M. ...	2	5,868	191	2,636	2,827	3,041	51·82
P. F. ...	3	5,958	146	2,945	3,091	2,867	48·12
H. S. ...	2	6,208	125	2,536	2,661	3,547	57·15
N. S. ...	1	6,050	148	2,690	2,838	3,212	53·09
Average	51·8

TABLE—conold.

Phosphorus balance.

Subject.	Age, years.	Intake in g.	Urinary excretion in g.	Fæcal excretion in g.	Total excretion in g.	Amount retained in g.	Percentage retained.
SOYA MILK :							
L. M. ...	2	2,625	182	1,191	1,373	1,252	47.69
A. C. ...	2	2,523	198	1,203	1,401	1,122	44.46
S. M. ...	2	2,870	167	1,355	1,522	1,348	46.97
P. F. ...	3	2,907	178	1,366	1,544	1,363	46.89
H. S. ...	2	2,904	191	1,283	1,474	1,430	49.23
N. S. ...	1	2,902	212	1,400	1,612	1,290	44.44
Average	46.6
Cow's MILK :							
L. M. ...	2	4,652	213	2,019	2,232	2,420	52.01
A. C. ...	2	4,652	168	2,116	2,284	2,368	50.90
S. M. ...	2	4,652	181	2,321	2,502	2,150	46.22
P. F. ...	3	4,724	202	2,150	2,352	2,372	50.21
H. S. ...	2	4,921	197	2,576	2,773	2,148	43.64
N. S. ...	1	4,797	167	2,118	2,285	2,512	52.36
Average	49.2

DISCUSSION.

The average figure for retention of protein is 60·7 per cent in the case of soya milk, while the corresponding figure for cow's milk is 70·3 per cent. This would show that once the subject is acclimatized to it, cow's milk protein is retained to a somewhat greater extent than that of soya-milk protein. The value for soya milk is, therefore, 86·3 per cent of that of cow's milk. This result is in agreement with the result of similar experiments on adult subjects and also on rats which showed that the proteins of soya milk were 90 per cent as good as the proteins of cow's milk regarding their nutritive value.

The data on calcium balance show that the average figures for calcium retention are 52·4 per cent in the case of soya milk and 51·8 per cent in the case of cow's milk. The calcium in cow's milk and soya milk is, therefore, equally well utilized. It has, however, to be noted that the calcium content of soya milk is only about 50 per cent of that of cow's milk.

The percentage utilization of phosphorus is 46·6 in the case of soya milk and 49·2 in the case of cow's milk. There is not much difference, therefore, in the availability of the phosphorus in the two milks.

As the subjects were very young and as the institution authorities were not in favour of any drastic alteration in the diet, it was not possible to carry out parallel trials with low nitrogen as well as low calcium and phosphorus diets. Although the reported figures are not absolute measures of the utilization of the three constituents, they are nevertheless of much relative importance as they relate to very young subjects.

One rather interesting feature emerges from the results. This relates to the high percentage of retention of the different constituents in the case of the children. This is particularly so with respect of calcium and phosphorus. Similar cases of high calcium and phosphorus retention have been observed by Stearns and Moore (1931), Hunscher *et al.* (1932) and Wang *et al.* (1928, 1930). It has been suggested that the previous nutritional status of the subjects may have had an important bearing on the retention of calcium and phosphorus. The fact that the children are actively growing and hence require these essential substances for their tissue growth might also provide a partial explanation.

SUMMARY.

1. Feeding experiments with soya milk were conducted with 30 children (1 to 3 years old). Children under one year received soya milk as the sole food, while older children received it as a supplement. It was observed that all the children readily took to soya milk. The growth rate was normal and no digestive disorder could be observed.

2. Comparative absorption and utilization of the proteins, calcium and phosphorus in cow's milk and soya milk was studied on select number of children. Soya-milk protein was 86 per cent as much utilizable as cow's milk protein. The utilization of calcium and phosphorus in the case of both the milks was fairly high, being over 50 per cent in the case of calcium and over 47 per cent in the case of phosphorus. There was practically no difference in the utilization of calcium and phosphorus in the two milks.

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THE LEVEL OF PROTEIN INTAKE AND THE QUALITY OF PROTEIN ON CALCIUM AND PHOSPHORUS ABSORPTION.

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INTRODUCTION.

THE beneficial effects of high protein intakes during periods of calcium deficiency have been observed by a number of workers (Mellanby, 1921, 1925; Pittman and Kunerth, 1939). Based on their earlier observations that the solubility of insoluble calcium salts is increased in solutions of amino acids, McCance *et al.* (1942) found that an increased protein intake results in a better calcium absorption and retention. While discussing their results they suggested the intake of a large quantity of protein for maximal calcium retention. Quantitative studies on the level of protein intake and the quality of protein in the diet on the utilization of dietary calcium and phosphorus were, therefore, undertaken by us and the results of such a study are presented in this paper.

EXPERIMENTAL.

The general plan of the experiment was to feed diets containing varying levels of protein (0, 5, 10 and 15 per cent levels) and fixed amounts of calcium and phosphorus to rats and study the amount of calcium and phosphorus absorbed and retained by the animals. Coagulated egg-white which contains very low amounts of calcium and phosphorus was taken as the source of protein in the diet. To

study the effect of quality of protein on calcium and phosphorus absorption, the egg-white was later replaced by an equivalent amount of a deficient protein-like gelatin, and the efficiency of calcium and phosphorus absorption was studied. A last experiment was also included in the series where the diet contained a single non-essential amino acid, like glycine, instead of a complete protein, like egg-white. The percentage of calcium and phosphorus assimilated was calculated in each case.

The experiment was performed on six healthy adult rats of about 150 g. in weight. Each experimental period consisted of feeding for one week, the last five days in each period being used for collection of excreta. Between successive experimental periods the rats were kept on a normal stock diet for four days to provide rest for the animals. The food intake of the animals was noted during each experimental period.

Calcium in the urine was estimated according to Shöhl and Pedley (1922). Calcium in the food and the faeces was estimated after ashing by the method of McCrudden (1911-12). The urine was analysed for phosphorus by the method of Fiske and Subba Row (1925). Perchloric acid was used for digestion of the urine samples. The food and faecal samples were ashed and later used for phosphorus estimation. Sodium acetate was added before ashing to fix the phosphorus.

The calcium and phosphorus balance data for the animals during the various periods and the percentage of calcium and phosphorus assimilated are given in the Table:—

TABLE.

(All values are expressed in mg. of calcium or phosphorus.)

Rat number.	CALCIUM.				PHOSPHORUS.			
	Intake.	Urinary excretion.	Faecal excretion.	Balance.	Intake.	Urinary excretion.	Faecal excretion.	Balance.
I. PROTEIN-FREE DIET:								
1	158.3	6.4	106.0	45.9	62.1	49.8	10.9	1.4
2	165.6	6.6	112.3	46.7	64.9	53.8	10.5	0.6
3	171.9	7.2	108.1	56.6	67.5	56.4	10.4	0.7
4	180.7	7.8	122.8	50.1	70.9	55.8	10.8	4.3
5	157.4	6.8	99.3	51.3	61.7	47.1	12.5	2.1
6	140.6	6.1	88.3	46.2	55.2	47.4	10.2	-2.4
Average	162.4	49.5	63.7	1.1

TABLE—contd.

Rat number.	CALCIUM.				PHOSPHORUS.			
	Intake.	Urinary excretion.	Fæcal excretion.	Balance.	Intake.	Urinary excretion.	Fæcal excretion.	Balance.
II. 5 PER CENT PROTEIN DIET :								
1	178.2	9.8	102.2	66.2	70.9	48.8	10.9	11.2
2	182.5	9.6	107.2	65.7	72.6	47.9	11.1	13.6
3	160.2	6.9	92.8	60.5	63.7	41.1	10.5	12.1
4	171.6	7.2	103.1	61.3	68.3	44.1	10.6	13.6
5	169.2	7.1	93.5	68.6	67.3	43.0	10.2	14.1
6	180.1	7.6	97.1	75.4	71.6	46.1	11.2	14.3
Average	173.6	66.3	69.6	13.2
III. 10 PER CENT PROTEIN DIET :								
1	175.9	9.5	94.0	72.4	70.3	33.6	10.9	25.8
2	168.6	8.4	86.1	74.1	67.3	32.5	11.7	23.1
3	170.8	8.2	89.0	73.6	68.1	31.3	11.2	25.6
4	181.6	8.1	95.2	78.3	72.5	31.0	11.4	30.1
5	156.7	8.5	79.1	69.1	62.5	28.4	11.4	22.7
6	176.1	9.9	96.1	70.1	70.3	34.4	10.8	25.1
Average	171.6	72.9	68.5	25.4
IV. 15 PER CENT PROTEIN DIET :								
1	180.8	10.4	78.0	92.4	72.3	24.6	11.6	36.1
2	176.2	9.2	76.4	90.6	70.5	24.7	10.9	34.9
3	196.3	9.6	88.6	98.1	78.6	26.8	11.5	40.3
4	185.4	10.6	82.9	91.9	74.1	22.7	10.9	40.5
5	170.7	10.1	72.4	88.2	68.2	24.6	10.5	33.1
6	181.6	9.5	79.3	92.8	72.6	26.5	10.5	35.6
Average	181.8	92.3	72.7	36.8

TABLE—contd.

Rat number.	CALCIUM.				PHOSPHORUS.			
	Intake.	Urinary excretion.	Fæcal excretion.	Balance.	Intake.	Urinary excretion.	Fæcal excretion.	Balance.

V. 15 PER CENT GELATIN DIET :

1	200.0	10.2	108.5	81.3	77.5	47.9	10.4	19.2
2	193.6	8.7	109.0	75.9	75.0	39.5	12.1	23.4
3	174.4	11.6	86.7	76.1	67.5	41.0	10.6	15.9
4	139.0	7.2	76.1	55.7	53.8	33.3	11.3	9.2
5	177.7	12.9	94.2	70.6	68.8	36.9	11.2	20.7
6	167.9	8.9	84.3	74.7	65.0	36.8	11.1	17.1
Average	175.4	72.4	67.9	17.6

VI. 5 PER CENT GLYCINE DIET :

1	154.2	10.6	94.7	48.9	61.3	50.9	9.8	0.6
2	159.7	9.8	94.2	55.7	63.5	49.5	10.0	4.0
3	143.3	8.9	85.7	48.7	57.0	48.7	9.8	-1.5
4	144.1	7.9	85.4	50.8	57.3	42.2	10.7	4.4
5	127.0	9.1	74.8	43.1	50.5	40.3	11.1	-0.9
6	148.1	10.4	87.9	49.8	58.9	45.6	11.6	1.7
Average	146.1	49.5	58.1	1.4

Note.—The composition of the diet given to the animals is given below :—

Corn starch 56 per cent, cane sugar 15 per cent, coco-nut oil 10 per cent, calcium-and-phosphorus-free salt mixture 4 per cent, and protein 15 per cent.

When the protein level had to be reduced to 10, 5 or 0 per cent, a requisite quantity of starch was added to make up the deficit quantity of protein. Yeast extract served as a source of vitamins of the B group. Vitamins A and D were supplied in the form of Adexolin.

TABLE—concl'd.

Average percentage utilization of calcium and phosphorus.

			Diet 1.	Diet 2.	Diet 3.	Diet 4.	Diet 5.	Diet 6.
Calcium	30.5	38.2	42.5	50.8	41.3	33.9
Phosphorus	1.7	19.0	37.1	50.6	25.9	2.4

DISCUSSION.

The data on the calcium and phosphorus balance of the animals under the various dietary regimes as represented in the Table clearly show the profound influence of dietary protein on calcium and phosphorus balance. On a protein-free diet calcium is utilized only to the extent of 30.5 per cent. There is practically no retention of phosphorus at all. In one case a negative phosphorus balance has been observed. At 5 and 10 per cent levels of protein intake, both calcium and phosphorus are better utilized. The effect is very marked, especially in the case of phosphorus. The phosphorus retentions at 5 and 10 per cent levels of protein are 19.0 and 37.1 per cent respectively while the corresponding figures for calcium are 38.2 and 42.5 per cent respectively. At a level of 15 per cent protein the percentages of calcium and phosphorus utilization are 50.8 and 50.6 respectively. These data, therefore, point out that for maximal calcium and phosphorus utilization, in addition to other beneficial dietary conditions, sufficient intake of protein must be ensured in the diet.

In the above experiments the beneficial response to higher protein intakes has been found to be very much more in the case of phosphorus than in the case of calcium. This differential response may perhaps be explained by the physiological interrelationships between phosphorus and protein metabolism. It is known that during times of protein deprivation the normal catabolic changes continue to take place resulting in the degradation of tissue proteins, especially those of the muscle. These proteins are specially rich in phosphorus. Hence, during the process of tissue breakdown, phosphorus is also catabolized and is voided. This fact, therefore, explains why phosphorus absorption responds better to higher protein intakes.

When egg-white protein is substituted by an equal amount of gelatin—a nutritionally inferior protein—it is seen that the percentage utilization of both calcium and phosphorus is decreased. A similar thing happens when a complete protein is replaced by a single amino acid like glycine. This shows that for efficient calcium utilization, not only should a sufficient intake of protein be ensured but also that the protein should contain all the essential amino acids. This observation raises the question as to whether the beneficial effect of protein in the diet on calcium and phosphorus utilization is solely due to the increased solubility of calcium salts in the amino acids provided by the protein as shown by the experiments of McCance *et al.* (*loc. cit.*) and Lehmann and Pollak (1942). Other hitherto unrecognized

physiological interrelationships of the metabolism of protein, calcium and phosphorus might provide an explanation.

SUMMARY.

Both calcium and phosphorus are better utilized on a high protein diet than on a low protein diet. Under the conditions of the above experiment there was a more marked effect on phosphorus utilization than on that of calcium. It has also been observed that the protein in the diet should contain all the essential amino acids so as to enhance the efficiency of calcium and phosphorus utilization. The physiological significance of the observed data has also been discussed.

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EFFECT OF TEMPERATURE ON THE GROWTH OF FISH SPOILAGE BACTERIA.*

BY

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It has been known for many years that fish should be kept frozen in cold storage to remain fresh. A number of workers have studied the bacteriological basis for this fact. Hess (1933, 1934) showed that bacterial decomposition of fish at 30°F. was only half as rapid as that at 36°F. This demonstrated the possibility of holding fish at 30°F. in cold storage instead of in melting ice. (Fish will not freeze at 30°F.). Stewart (1932, 1933, 1934) showed that fish spoilage bacteria dropped in numbers at -12°C. and -6°C. rapidly at first, then gradually. At -2°C. a drop occurred at first, then the numbers rose until the fish spoiled. Gibbons (1934) examined bacteriologically frozen blocks of fish after varying periods of storage at -5°C. to -18°C. for a year. Total bacterial counts showed that after a decrease due to freezing there was an increase of psychrophilic organisms after fifty weeks. Bedford (1933) examined the temperature of growth for various marine bacteria and found many grew at very low temperatures, but found their optimum at 20°C. to 25°C.

EXPERIMENTAL.

The experiment herein reported was designed to determine the rate at which micro-organisms multiply in fish tissue at 37°C., 21°C., 0.5°C. and -2°C.

A specimen of 'orange rock fish' was brought to the laboratory from a boat which was landing its catch. A representative sample of slime was collected from the surface of the fish in a sterile test-tube. The fish was quite fresh at the time it was brought to the laboratory.

In order to get rid of surface contamination on the outside of the fish, the skin was swabbed with alcohol, then flamed until slightly charred. Skin and scales were removed aseptically.

* Published with the kind permission of the Director of Industries and Commerce, Madras.

One pound of flesh was removed aseptically and this was transferred to two sterile Waring blenders. To each of these was added 225 c.c. of sterile sea-water and the mixture was well minced. During the process of blending 25 c.c. of slime was added to the mixture by means of a sterile pipette. The blending was carried out for 5 minutes and then the mixture transferred to sterile bottles.

Original plate counts were made and the bottles containing the minced flesh were stored at 37°C., 21°C., 0.5°C. and -2°C. Periodic counts were made thereafter until the flesh was judged spoiled by its odour. The diluent for the platings was autoclaved sea-water and the medium was nutrient agar made up with sea-water. Platings were made in quadruplicate and duplicates were incubated at 21°C. and 37°C. The counting was made after six days' incubation.

A number of these organisms growing on these plates were identified to the genus by determining Gram-stain, morphology of colony and motility.

RESULTS.

The results are shown in Graphs 1 to 4.

Graph 1 shows the curve obtained at 37°C. incubation. The growth of bacteria to start with is slow and the lag phase lasts for about eight hours after which there is a sudden increase in the count. The logarithmic growth phase extends over a period of eight hours after which the number begins to fall and at the end of 24 hours the flesh begins to smell putrid. It is concluded that the fish, once it is out of water, can hardly be kept for twelve hours in good condition at a temperature of 37°C.

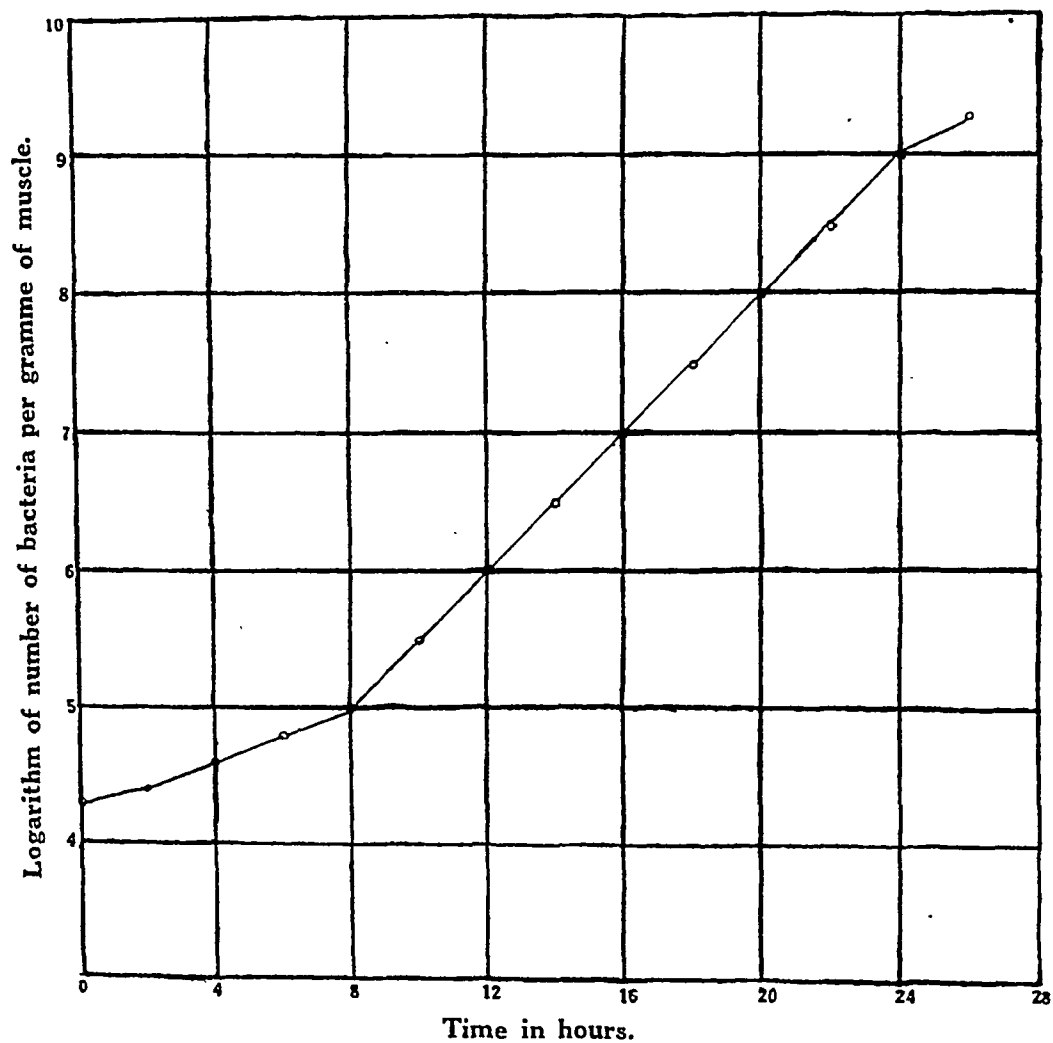
Graph 2 shows the curve obtained at 21°C. incubation. The lag phase is prolonged and it lasts for about 16 hours. During this period there is a very gradual rise in the count, but the actual logarithmic growth sets in after about twenty-eight hours. This phase is very short, lasting about twelve hours when the maximum count is obtained and the muscle undergoes rapid deterioration during this phase. After 40 hours the muscle begins to leave a putrid smell. Fish at 21°C. will be in an inedible condition after about twenty-four hours.

Graph 3 shows the curve obtained at 0.5°C. The muscle at this temperature does not freeze. There is no initial fall in numbers. The lag phase lasts for over six days during which period the flesh of the muscle has a fishy odour. On the seventh day there is an increase in the count and the logarithmic growth phase sets in. From this period onwards there is a very rapid rise in the count and the muscle undergoes very rapid deterioration and on the twelfth day the count is in billions. At the temperature of 0.5°C., which may be taken as the temperature in the fish hold, the fish can be kept in good condition for eight days.

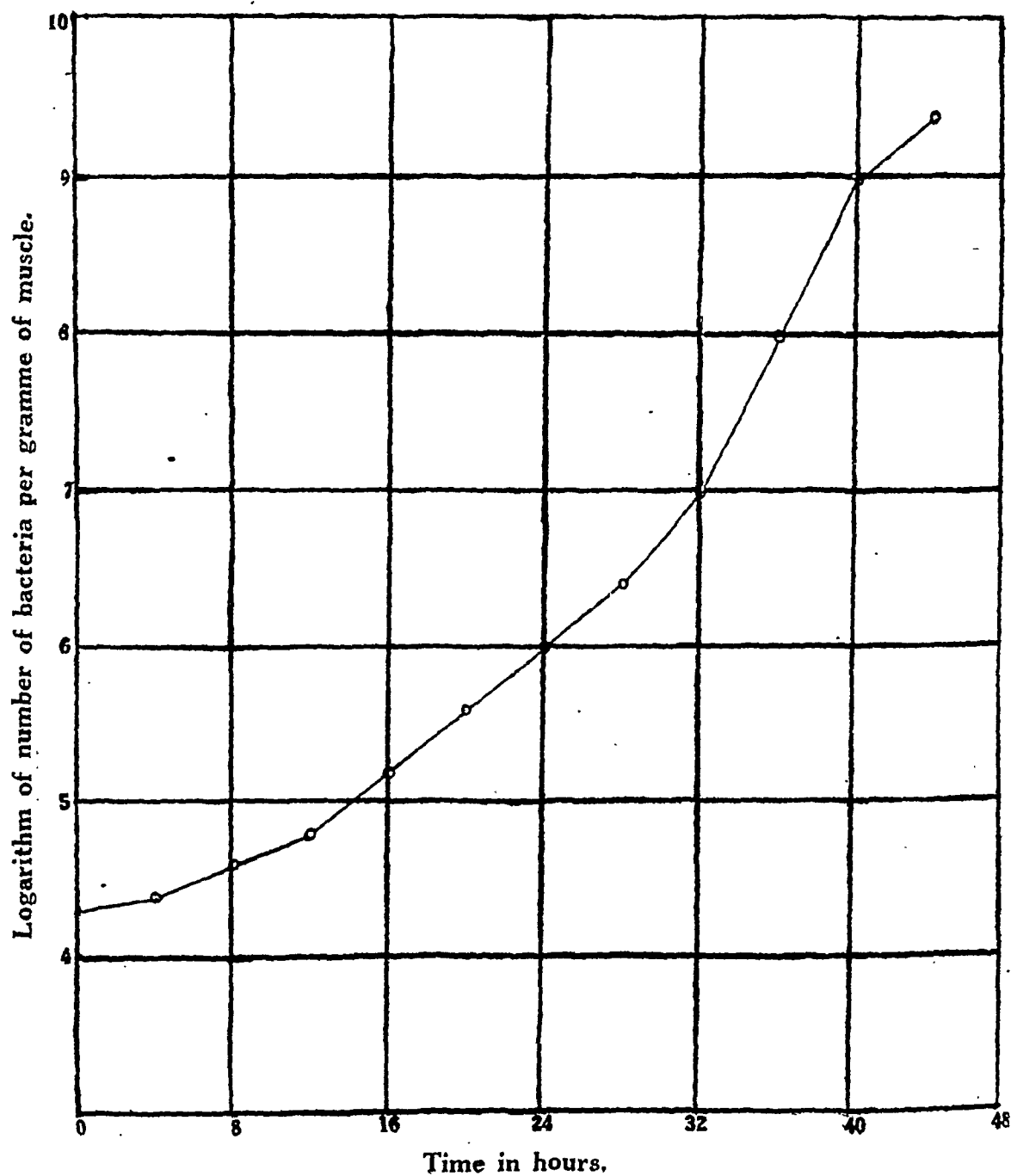
Graph 4 shows the curve obtained at -2°C. This was the most interesting phase of the work. It was found that there was an initial fall in numbers. The muscle was frozen solid after ten hours' incubation. This initial fall continued until the eighth day after which the counts began to rise gradually. In other words the lag phase lasted eight days during which period there was a slight fall in initial numbers. The gradual increase continued until the twenty-eighth day

GRAPH 1.

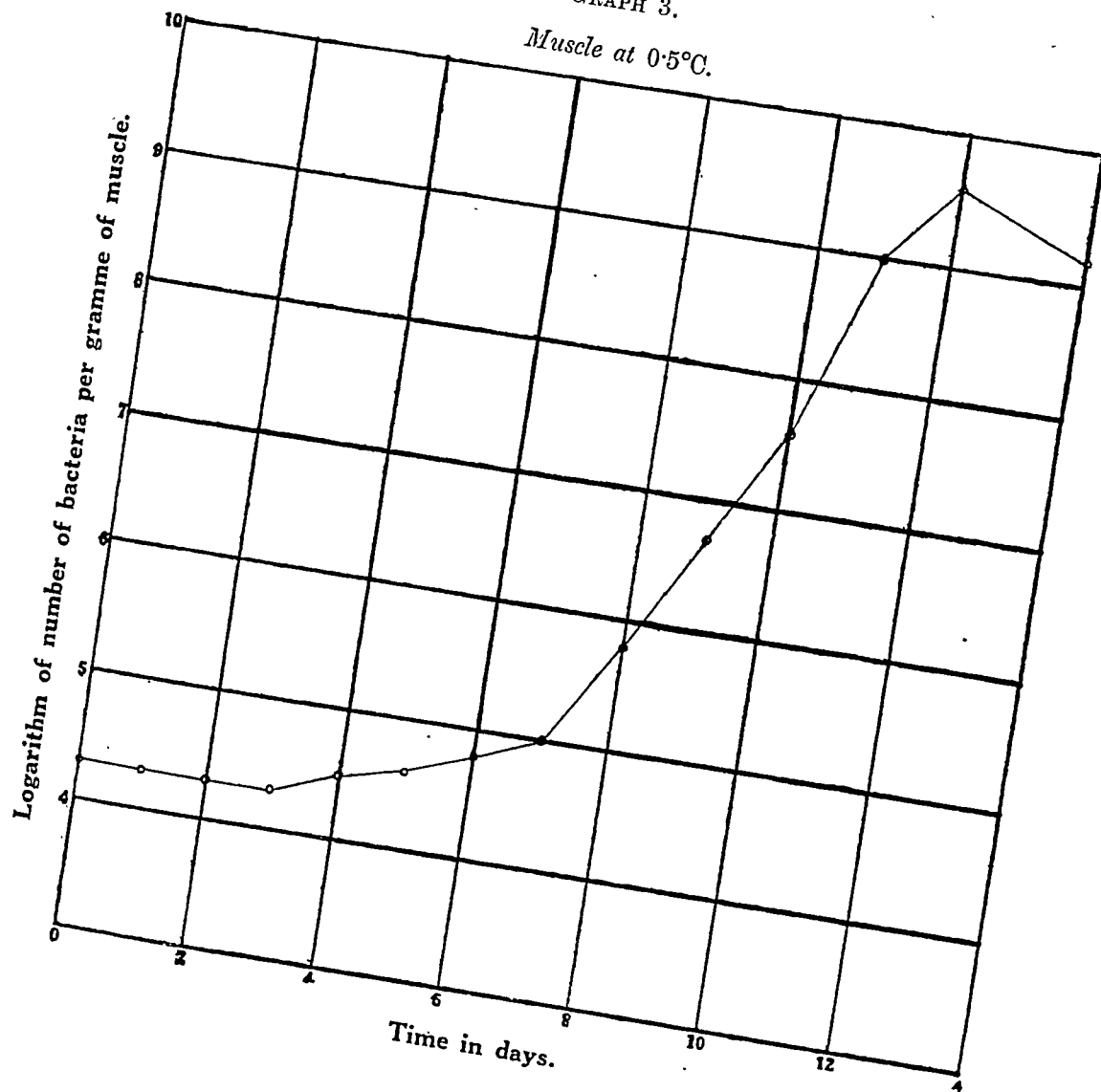
Muscle at 37°C.



GRAPH 2.

Muscle at 21°C.

GRAPH 3.
Muscle at 0.5°C.



The following genera were recorded:—

		37°C.	21°C.	0·5°C.	—2°C.
<i>Micrococcus</i>	...	a	a	a	a
<i>Sarcina</i>	...	—	b	—	—
<i>Pseudomonas</i>	...	—	b	b	a
<i>Achromobacter</i>	...	a	b	b	—
<i>Flavobacterium</i>	...	—	a	b	b
<i>Bacillus</i>	...	a	—	—	—
<i>Serratia</i>	...	—	a	b	—
<i>Kurthia</i>	...	—	—	b	—

a = Abundant.

b = Less abundant.

— = Absent.

It will be seen from the above data that there is a variation in the microbial flora at these different temperatures. While *micrococcus* is present at all the temperatures of incubation, other genera are totally absent. *Pseudomonas* was never met with at 37°C. incubation, while at 21°C. there were a few and at 0·5°C. and —2°C. it was the most predominant. *Serratia* was absent at 37°C., met with at 21°C. and a few at 0·5°C., and absent at —2°C. *Kurthia* was met with only in one instance and that was at 0·5°C. *Bacillus* was met with only at 37°C. *Achromobacter* was found in abundance at 37°C., 21°C. and 0·5°C. but totally absent at —2°C. In fact only two genera were represented at —2°C., namely, *micrococcus* and *pseudomonas*. This incidentally furnishes us the optimum temperature of growth of some of these organisms, which also accounts for the rapid or slow spoilage of the muscle at the varying temperatures.

DISCUSSION.

There is considerable increase in the lag phase between the temperatures of 0·5°C. and —2°C. This increase is due to the freezing of the muscle tissue at the temperature of —2°C. This temperature, however, is not lethal to the bacteria. It takes them some time to adapt themselves to the new environment, which is about ten days in the present experiment after which they begin to multiply gradually. Once multiplication starts, there is a steady rise in numbers.

The temperature of —2°C. can be used where storage for not more than 30 days are required. It must, however, be borne in mind that at this temperature bacterial growth does not cease and one ought to be extremely careful in using this temperature for : e.

SUMMARY.

1. Fish muscle undergoes rapid deterioration at 37°C. and 21°C. and gets putrid in a period of sixteen and forty hours respectively.
2. Muscle at 0.5°C. has an extended lag phase and is in good condition for eight to ten days.
3. Muscle at -2°C. is in excellent condition for nearly thirty days.
4. There is a variation in the microbial flora growing at these different temperatures.

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STUDY OF THE MECHANISM OF BIOSYNTHESIS OF ASCORBIC ACID DURING GERMINATION.

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INTRODUCTION.

In many parts of India germinated pulses are used as common articles of food. That a large amount of ascorbic acid is produced in pulses during germination has been shown by Guha and Ghosh (1934), Ahmad (1935), Rudra (1938), Ahmad and Muthana (1940), Bhagvat and Rao (1942), Fa and Roy (1944), and others.

The nature of the precursor required for the synthesis of ascorbic acid in plant and animal tissues has been studied by Ray (1934) and Guha and Ghosh (1934a, 1935, 1936). The effect of certain physical and chemical agents has also been studied but the results obtained show great discrepancy. Moreover, most of the previous works have been conducted by Tillman's titration method which does not give accurate results in micro-determination. The newly devised photo-electric method is more accurate and reliable than the ordinary titration method in this respect and it was, therefore, felt that the re-investigation of the biosynthesis of ascorbic acid by the newly devised method and the study of the effects of different physical and chemical agents on the production of this acid during germination may give some idea as to the possible mechanism of biosynthesis of this acid.

EXPERIMENTAL.

Dry legumes were washed with re-distilled water and soaked in solution used for germination, for 6 to 12 hours. After the soaking was complete, they were scattered on ashless filter-paper sterilized in Petri-dishes and covered with glass-lid to prevent evaporation. The dishes were placed on a table near a glass window

facing north. For germination in the dark, the Petri-dishes were kept in a wooden box painted black inside and outside, and having a zigzag outlet for the circulation of the air. The dishes of each set of experiments were subjected to identical conditions of temperature and illumination. The seeds were kept moist during germination by frequent addition of the solution.

Composition of the solution of the culture medium used.—In certain experiments, Knoop's solution was used in medium of gelatin, agar agar and sand. Knoop's solution contained :—

Calcium nitrate	0.8 g.
Potassium nitrate	0.2 g.
Potassium di-hydrogen phosphate	0.2 g.
Magnesium sulphate	0.2 g.
Ferric phosphate	Trace.
Water (re-distilled)	10,000 c.c.

Method of analysis.—One per cent metaphosphoric acid solution was used as the extracting liquid and ascorbic acid was estimated with 2 : 6 di-chlorophenol indo-phenol by means of Klett Summerson photo-electric colorimeter by the modified method of Loeffler and Ponting (1942) with filter 520. The calibration factor was determined with solutions of pure synthetic ascorbic acid in freshly prepared 1 per cent metaphosphoric acid solution. Dehydro-ascorbic acid was estimated after reduction with H_2S and the removal of the extra H_2S with CO_2 or N_2 according to the technique of Harris and Oliver (1942).

RESULTS AND DISCUSSION.

Effect of germination period on ascorbic acid synthesis.—Pea (*Pisum sativum*), mung (*Phaseolus radiatus*), cowpea (*Vigna catieng*) and kalai (*Phaseolus mungo*) were germinated with distilled water in diffused light and in darkness at room temperature and their vitamin C contents were estimated every day. This experiment was conducted for a period of eight days. It will be observed from the data presented in Table I that the vitamin C contents of the dry pea and mung are 8.75 mg. and 11.25 mg. respectively per 100 g. dry weight, whereas those of cowpea and kalai are *nil*. As the period of germination increased the ascorbic-acid content also increased until it reached a maximum on the 3rd day in the case of pea and kalai and on the 5th and 6th days in case of mung and cowpea respectively both in light and darkness. The maximum ascorbic-acid value of pea was found to be 25 mg. in light and 25.5 mg. in dark, of mung 62.5 mg. in light and 60 mg. in dark, of cowpea 54 mg. in light and 53 mg. in dark and of kalai 40 mg. in light and 42 mg. in dark per 100 g. dry basis. After reaching a maximum value the ascorbic-acid content showed a sudden fall and thereafter began to decline keeping a lower but a constant value. Ray (*loc. cit.*) working with pea seedlings and Fa and Roy (*loc. cit.*) working with soya bean observed similar fall in ascorbic-acid values after a peak.

EFFECT OF LIGHT.

Table I shows that there is no significant difference in the synthesis of ascorbic acid in diffused light and in darkness. This indicates that light does not play any

direct rôle in the biosynthesis of ascorbic acid in germinating legumes. Roy, Bose and Guha (1944) working with soya bean and 'kancha mung' observed an increase in the content of the indophenol-reducing substance similar to ascorbic acid on exposure to sun and ultra-violet light, but as a result of bioassay, they have shown that the substance produced under the stimulation of sun and ultra-violet light is not identical with ascorbic acid. The reducing substance produced under darkness was found to be mostly ascorbic acid. Many other workers have observed similar increase in the indophenol-reducing substance in plants and fruits under the influence of direct sunlight but that this increase is not directly related to photosynthesis will be evident from other experiments reported in this paper.

TABLE I.

Ascorbic-acid synthesis in germinating legumes.

(The ascorbic-acid values are expressed in mg. per 100 g. dry weight.)

Name of the legume.	Germinated in :	PERIODS OF GERMINATION EXPRESSED IN HOURS :								
		0	24	48	72	96	120	144	168	192
Pea	Light ...	8.75	16.20	21.25	25.0	12.5	11.25	11.0	10.5	10.0
	Dark ...	8.75	16.25	20.0	25.5	12.5	12.0	10.5	10.0	9.0
Mung	Light ...	11.25	25.0	43.75	48.0	54.0	62.5	35.0	32.0	32.0
	Dark ...	11.25	25.0	42.5	49.0	53.0	60.0	36.0	34.0	32.0
Cowpea	Light ...	Nil.	5.0	27.5	37.5	48.0	53.0	54.0	32.0	30.0
	Dark ...	Nil.	5.0	27.5	37.0	49.0	52.0	53.0	31.0	31.0
Kalai	Light ...	Nil.	7.5	27.0	40.0	20.0	19.5	18.0	17.5	16.0
	Dark ...	Nil.	7.5	26.5	42.0	21.0	19.0	18.0	17.0	17.0

RÔLE OF CHLOROPHYLL ON THE BIOSYNTHESIS OF ASCORBIC ACID.

In the above experiment it was observed that the seeds germinated in darkness were white in colour, whereas those germinated in diffused light were green in colour. As the ascorbic-acid contents in the two stages of germination were almost similar, it may, therefore, be interpreted that chlorophyll does not play any direct rôle on ascorbic-acid synthesis. This independence of ascorbic-acid synthesis and the presence of chlorophyll was also observed by Giroud *et al.* (1934), Randoïn *et al.* (1935) and Reid (1937) in their experiments with growing plants. Weber (1910) showed that the seedlings of various species grown at a low temperature

are inhibited in the production of chlorophyll but the ascorbic-acid content was higher than those kept at ordinary temperature and in light. The present investigation confirms the earlier findings of other workers that for the biosynthesis of ascorbic acid the presence of chlorophyll is not required.

DEHYDRO-ASCORBIC ACID CONTENT OF GERMINATING LEGUMES.

In our previous experiments it was shown that as the period of germination proceeded the ascorbic-acid contents suddenly declined after reaching a maximum value. In order to ascertain whether this sudden fall in the values was due to the conversion of the ascorbic acid to the dehydro stage, fresh samples of mung and cowpea were germinated both in light and darkness for a period of 120 hours according to the technique employed in the previous experiment and their ascorbic-acid and dehydro-ascorbic acid contents were estimated every 24 hours. The results of the experiment are presented in Table II from which it will be observed that at the early stage of germination the dehydro-ascorbic acid was not present in a measurable quantity but in the later period when the ascorbic-acid content

TABLE II.

Ascorbic-acid and dehydro-ascorbic acid contents of germinated legumes.

(Values expressed as mg./100 g. dry weight.)

Name of legume.	Period of germination in hours.	LIGHT.		DARK.	
		A.A.	D.-A.A.	A.A.	D.-A.A.
Mung ...	0	10.0	0.0	10.0	0.0
	24	22.5	0.0	22.0	0.0
	48	41.0	1.0	41.5	1.0
	72	40.0	1.0	40.5	1.0
	96	22.0	1.5	23.0	2.0
	120	21.0	2.0	22.0	2.0
Cowpea ...	0	0.0	0.0	0.0	0.0
	24	7.0	0.0	7.2	0.0
	48	29.0	0.0	28.0	0.0
	72	38.0	1.0	39.0	1.0
	96	52.5	1.0	52.0	1.0
	120	30.0	2.0	31.0	2.0

A.A. and D.-A.A. denote ascorbic acid and dehydro-ascorbic acid respectively.

was declining a small amount of the former could be detected. It is evident from these results that the sudden decline in the ascorbic-acid content during the process of germination is not due to conversion of this acid to the dehydro stage.

DISTRIBUTION OF ASCORBIC ACID IN COTYLEDON AND EMBRYO.

In our previous experiment it has been shown that light and chlorophyll do not play any rôle in the synthesis of ascorbic acid. Perhaps this synthesis depends on some precursors of carbohydrate nature furnished by the reserves of the seed. In order to get some information in this matter, fresh batches of cowpea, pea, mung and kalai seeds were germinated for a period of six days and after every 24 hours the embryos and cotyledons were separated and their ascorbic-acid content measured. It will be observed from Table III that the ascorbic-acid content of the cotyledons and embryos of the four pulses after first 24 hours' germination is between 60 to 70 per cent and 30 to 40 per cent respectively. As germination proceeded the value in the cotyledons began to decline and reached the limit of 17 to 27·5 per cent in 144 hours and that of the embryos began to increase and reached the limit of 74·5 to 83 per cent in the same period. This shows that at the initial stage the ascorbic acid is synthesized from some precursors in the cotyledon for the development of the embryos which require ascorbic acid at their early stages and at the later stages these embryos can synthesize this compound on their own. Bhagvat and Rao (*loc. cit.*) also found similar results in their study on vitamin C produced in the different grains during the process of germination.

TABLE III.

Ascorbic acid of the cotyledon and embryo expressed as percentage of the total amount synthesized in the seed.

Period of germination in hours.	COWPEA.		MUNG.		PEA.		KALAI.	
	C	E	C	E	C	E	C	E
24	67·5	32·5	65·0	35·0	60·5	39·5	58·5	41·5
48	55·5	44·5	48·0	52·0	45·0	55·0	41·0	59·0
72	43·0	57·0	35·0	65·0	32·0	68·0	30·0	70·0
96	33·0	67·0	29·0	71·0	25·0	75·0	23·0	77·0
120	30·0	70·0	22·0	78·0	21·0	79·0	20·0	80·0
144	25·5	74·5	20·0	80·0	19·0	81·0	17·0	83·0

C and E denote cotyledon and embryo respectively.

INFLUENCE OF THE SEED COAT ON THE SYNTHESIS OF ASCORBIC ACID
IN GERMINATING LEGUMES.

Cowpea and mung were germinated, some with seed coat intact and others without seed coat. Table IV shows that there is no significant difference in the ascorbic-acid content in the earlier period of germination but in the later periods, i.e. on the 5th and 6th days of germination, the ascorbic-acid content of the seed germinated without coat was somewhat lower than that of the legumes germinated with the coat. Altogether it may be said that the seed coat has no significant effect on the synthesis of ascorbic acid in the germinating seeds.

TABLE IV.

Influence of the seed coat on the synthesis of ascorbic acid in germinating legumes.
(Values are expressed in mg. per 100 g. dry weight.)

Name of the legume.				PERIOD OF GERMINATION IN HOURS :					
				24	48	72	96	120	144
Cowpea	...	With seed coat	...	6.0	29.0	38.0	55.0	34.0	33.0
		Without seed coat	...	6.0	28.0	39.0	54.0	30.0	25.0
Mung	...	With seed coat	...	27.0	44.5	49.0	56.0	64.0	36.0
		Without seed coat	...	27.5	44.0	49.0	55.0	63.0	30.0

*Rôle of trace elements on the biosynthesis of ascorbic acid in
germinating legumes.*

Different batches of mung seeds were germinated in solutions containing 0, 10, 100 and 1,000 p.p.m. of iron, copper and manganese as ferric nitrate, copper nitrate and manganese chloride (since manganese nitrate was not available, chloride was used) both in light and darkness and after 48 hours of germination their ascorbic-acid contents were estimated. Since the values of the wet weight, dry weight and the ascorbic-acid content of the legumes germinated in light as well as in darkness were almost of the same order, the results of light germination have only been presented in Table V. It will be observed that in all three cases the ascorbic-acid synthesis is augmented up to a certain concentration of the solution above which it is retarded. The maximum ascorbic-acid synthesis was found near the concentration of 10 p.p.m. and the maximum values were found to be 59 mg., 65 mg. and 79 mg. per 100 g. dry weight respectively. By further investigation with concentrations of 5, 10 and 15 p.p.m. it was again found that the maximum ascorbic-acid synthesis takes place at the concentration of 10 p.p.m., although in case of manganese a slight tendency for further increase in the values was observed when the concentration was raised to 15 p.p.m. From the survey of the data presented in Table V it is evident that manganese favours the ascorbic-acid synthesis best, then comes copper and then iron, in this respect.

TABLE V.

Rôle of trace elements on the biosynthesis of ascorbic acid in mung during germination in light.

Nature of the salt.	Concentration in p.p.m.*	Original weight in g.	Wet weight in g.	Dry weight in g.	Ascorbic acid in mg./100 g. dry weight.
SERIES A:					
Ferric nitrate ...	0	2	6.5	1.64	43
	10	2	6.8	1.61	59
	100	2	6.5	1.75	40.5
	1,000	2	6.0	1.92	36
Copper nitrate ...	0	2	10.2	1.75	44
	10	2	11.6	1.65	65
	100	2	10.0	1.72	49
	1,000	2	7.0	1.95	24
Manganese chloride	0	2	8.2	1.62	44
	10	2	8.0	1.60	79
	100	2	8.0	1.60	73
	1,000	2	7.4	1.62	61
SERIES B:					
Ferric nitrate ...	5	2	11.7	1.61	55
	10	2	12.0	1.60	61
	15	2	11.8	1.60	58
Copper nitrate ...	5	2	11.8	1.62	61
	10	2	12.2	1.61	66
	15	2	12.0	1.60	65
Manganese chloride	5	2	11.5	1.61	74
	10	2	12.0	1.60	80
	15	2	12.2	1.60	82

* Refers to the concentrations of iron, copper and manganese as ferric nitrate, copper nitrate and manganese chloride.

The importance of manganese in the biosynthesis of ascorbic acid in both plant and animal kingdom has been indicated by many workers by observing its accelerating effect and correlation on the production of the above acid (Rudra, 1938, 1939, 1939a; Hester, 1941). In the present investigation similar accelerating effect on the biosynthesis of ascorbic acid was produced by iron and copper salts also, although to a lesser extent. So in the light of these observations manganese cannot be regarded as the only element to act as a catalyser as postulated by Rudra and others (*loc. cit.*). The ascorbic acid of plant and seed depends not only on the manganese content of the soil but also on other essential elements present therein. It is also interesting to note here that copper which catalyses the destruction of ascorbic acid in *in vitro* experiments helps in its production when present in soil or plant.

EFFECT OF DIFFERENT SUGARS ON THE BIOSYNTHESIS OF ASCORBIC ACID.

Cowpea embryo seedlings ten in number were separated from the cotyledons after eight hours of soaking in distilled water and then cultured separately both in light and darkness with 5 per cent solutions of glucose, fructose, galactose and sucrose and with 0.05, 2.5 and 5 per cent solutions of mannose in Knoop's solution in a medium of (a) 20 per cent gelatin, (b) 1 per cent agar agar and (c) acid-washed white sand, and their wet weight and ascorbic-acid content were measured after four days according to the technique followed by Ray (*loc. cit.*) with some modifications. One set of cowpea seedlings were allowed to grow along with cotyledons in a medium containing 20 per cent gelatin and separated after incubation period of four days. As the wet weight and the ascorbic-acid content of the seedlings cultured in different sugars were the same both in light and darkness, the values of those cultured in the dark have, therefore, been omitted. The results presented in Table VI show that the seedlings grown in nutrient cultures with different sugars produced considerably higher amount of ascorbic acid than those grown in cultures without sugars. The ascorbic-acid content of the seedlings grown in sucrose, glucose, fructose and galactose was almost of the same order, whereas those grown in mannose showed higher values. Small concentration as 0.05 per cent mannose solution produced greater amount of ascorbic acid than 5 per cent solutions of other sugars studied here. So mannose may be regarded as the most efficient of the five sugars under investigation in favouring biosynthesis of ascorbic acid.

It was also found that seedlings excised after eight hours and grown in nutrient solution without any sugar showed maximum growth but least ascorbic-acid content, whereas those grown in a medium containing 5 per cent mannose solution showed minimum growth but maximum ascorbic-acid content. It was also further observed that as the concentration of the mannose solution in the medium decreased the ascorbic-acid content decreased in a similar manner but the growth increased. Although mannose favours the ascorbic-acid synthesis, it inhibits the growth to a great extent.

TABLE VI.

Effect of different sugars on the synthesis of ascorbic acid in cowpea seedlings during germination in light.

Name of sugar added.	NAME OF THE CULTURE MEDIUM:					
	20 PER CENT GELATIN.		1 PER CENT AGAR AGAR.		WHITE SAND.	
	Wet weight in g.	Ascorbic acid in mg./100 g. wet weight.	Wet weight in g.	Ascorbic acid in mg./100 g. wet weight.	Wet weight in g.	Ascorbic acid in mg./100 g. wet weight.
Nil	0.38	2.4	0.6	4.8	0.56	2.4
Glucose (5 per cent) ...	0.22	31.0	0.42	35.0	0.32	30.6
Fructose (5 per cent) ...	0.27	45.0	0.46	44.0	0.47	44.0
Galactose (5 per cent) ...	0.28	34.0	0.48	34.0	0.40	35.0
Sucrose (5 per cent) ...	0.25	42.0	0.40	41.0	0.36	42.0
Mannose (5 per cent) ...	0.16	72.0	0.31	66.0	0.29	68.0
Mannose (2.5 per cent) ...	0.18	60.0	0.32	64.0	0.29	63.0
Mannose (0.05 per cent) ...	0.22	44.0	0.41	45.0	0.43	43.0
Naturally grown embryos* ...	1.8	85.0

* Embryos grown along with the cotyledons for four days in the medium without any sugar and then excised.

It was also interesting to note that the embryos allowed to grow along with the cotyledons in gelatin medium without any sugar and then excised after incubation period of four days, showed higher concentration of ascorbic acid than those which were separated from the cotyledons after eight hours and then cultured for four days in the same medium. The present observations are in contradiction to those of Ray (*loc. cit.*) who observed similar concentration of ascorbic acid in both cases. The lower value of ascorbic acid of the embryos separated after eight hours is probably due to the fact that cotyledon furnishes some sugars which act as precursors for ascorbic-acid synthesis, and when the embryos were separated from the cotyledons at the early stage of germination after eight hours, sufficient quantity of the precursor sugars could not be transformed to ascorbic acid and only a small portion of the ascorbic acid thus synthesized from precursor sugar of cotyledon is transferred to the embryos during this small period of eight hours' germination. Although the embryos separated from the cotyledons after eight hours of soaking

do not contain large store of ascorbic acid within but they possess the capacity to synthesize this acid from different sugars as mannose, glucose, fructose, galactose and sucrose, as is evident from the data presented in Table VI.

It has previously been reported that both in light and darkness the growth of the seedlings was almost the same and this shows that the growth can take place even in the absence of light and chlorophyll. In such cases ascorbic acid seems to play some definite rôle in the development of the seedlings. It may be postulated that growth proceeds at the expense of ascorbic acid, the production of which, on the other hand, depends on the reserve of the precursor sugars present in the cotyledon and is independent of light and chlorophyll as discussed above.

SUMMARY.

1. On germination ascorbic acid is abundantly synthesized in legumes, such as pea, cowpea, mung and kalai. The value reaches the maximum on the third to sixth days of germination, then suddenly decreases and keeps lower but constant for further two to five days.

2. Chlorophyll and the seed coat do not seem to have any influence on the biosynthesis of ascorbic acid in germinating legumes.

3. Dehydro-ascorbic acid content of the dry and germinated legumes was found to be negligible.

4. In the earlier stage of germination most of the ascorbic acid was found in the cotyledon but as the germination proceeded a major portion was found in the seedlings.

5. Solutions of copper nitrate, iron nitrate and manganese chloride were found to augment the biosynthesis of ascorbic acid in germinating mung, and maximum value was obtained when the concentration of iron, copper or manganese were in the region of 10 p.p.m.

6. Study of the biosynthesis of ascorbic acid in growing cowpea embryos cultured in different sugars in both light and darkness shows that mannose is one of the best precursors of ascorbic-acid synthesis. An inverse relationship between the ascorbic-acid content and the growth was observed when the embryos were cultured in mannose solution of varying strength.

7. Light does not seem to play a direct rôle on the biosynthesis of ascorbic acid.

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It is well known that the liver is almost invariably enlarged in kala-azar, though this *per se* does not mean that there is any hepatic dysfunction. The pathological changes that occur in the liver, viz. parasitization and marked proliferation of the Kupffer cells that lead to a marked alteration of the pattern of the hepatic lobule, variable degrees of fatty degeneration of the parenchyma cells, increase of the connective tissue in the late stages, vascular engorgement to a variable degree, etc., however, show that the liver is markedly involved in the disease process of visceral leishmaniasis. The hepatic function tests that have been carried out in kala-azar in the past, viz. l  vulose-tolerance test (Napier and Sharma, 1933), Takata-Ara test (Pai, 1941 ; Chaudhuri and Rai Chaudhuri, 1943), the van den Bergh test (Napier, *loc. cit.*) and the changes in the plasma-proteins have also indicated the probability of hepatic dysfunction in kala-azar.

In our study of the hepatic function in kala-azar the following six tests were selected : (intravenous) hippuric-acid synthesis test, prothrombin-time estimation, van den Bergh test, serum-colloidal gold reaction, Takata-Ara test and the study of the serum-proteins. The hippuric-acid test, the estimation of plasma-bilirubin and serum-proteins and the colloidal-gold test were selected for study following the suggestions of Mateer *et al.* (1943), Higgins *et al.* (1944) and MacLagan (1944, 1944a), who have shown the efficiency of these tests in the diagnosis of hepatic disorder by comparison with normal controls and other diseases. The other two tests were selected because they were thought to be particularly suitable for the study of the hepatic function in this disease. The estimation of prothrombin time is regarded as a very sensitive liver-function test (Beaumont and Dodds, 1944) and it was carried out in the expectation that it might throw some light on the h  morrhagic tendency in kala-azar as well. The Takata-Ara test depends on the alterations in the plasma-proteins which are known to occur in kala-azar. In all the cases investigated the clinical diagnosis of kala-azar was confirmed by finding the parasite and/or the aldehyde and the complement-fixation tests.

The hippuric-acid synthesis test.—This test was carried out according to the method of Quick *et al.* (1938), and the solution of sodium benzoate administered intravenously. The one-hour sample of urine was tested for the quantity of hippuric acid present according to the method of Quick (1933) or Weichselbaum and Probstein (1939). Table I shows the results obtained in a series of 55 cases :—

TABLE I.

Amount of hippuric acid excreted in g.	Number of cases.
0.00 to 0.1	3
0.11 to 0.2	13
0.21 to 0.3	2
0.31 to 0.4	10
0.41 to 0.5	3
0.51 to 0.6	2
0.61 to 0.7	11
0.71 to 0.8	6
0.81 to 0.9	4
0.91 to 1.0	1

It will be seen from Protocol I that 39 out of 55 cases showed lowered excretion of hippuric acid, i.e. 70.9 per cent of the cases showed positive reaction with this test (hippuric-acid excretion below 0.7 g.).

The importance of the body-size and its relation to the amount of excretion of hippuric acid has recently been studied by Scurry and Field (1943). They found that a definite correlation exists between the hippuric-acid excretion and the body-size as judged by the body-weight or the body-surface, the correlation being almost identical for the two factors. They analysed the data statistically and arrived at the following tentative formula for the prediction of normal excretion :—

Hippuric acid in g. = $0.34 \text{ plus } (0.00668 \times \text{weight in lb.})$

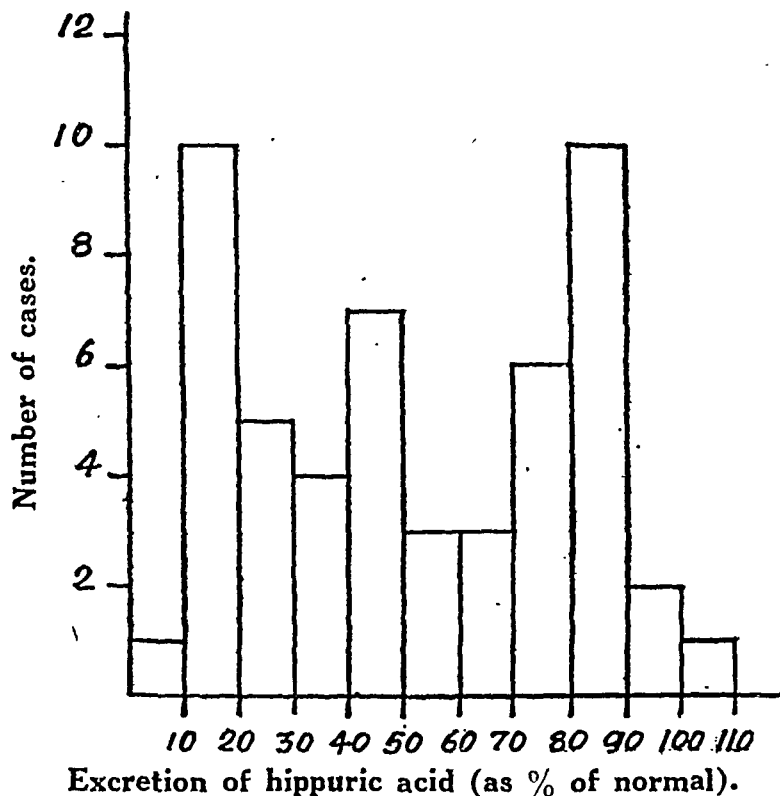
= $(0.837 \times \text{body-surface in sq. metres}) - 0.163$.

They obtained results between 72.8 and 125 per cent of the predicted amount in normal cases and contend that 70 per cent is the border-line of normal excretion except in children and very old people. In the present series the results obtained according to this formula in relation to the body-weight have been expressed as the percentage of calculated normal excretion in Table II, Graph 1 and Protocol I.

TABLE II.

Per cent of normal excretion.	Number of cases.
0 to 10 ...	1
11 to 20 ...	10
21 to 30 ...	5
31 to 40 ...	4
41 to 50 ...	7
51 to 60 ...	3
61 to 70 ...	3
71 to 80 ...	6
81 to 90 ...	10
91 to 100 ...	2
101 to 110 ...	1

GRAPH 1.



Hippuric-acid excretion in kala-azar (Quick's intravenous method).

According to this formula 33 out of 53 patients, i.e. 62.3 per cent, showed excretion below 70 per cent of the predicted normal and, therefore, showed signs of hepatic dysfunction.

In 18 out of 55 cases (32.7 per cent) no precipitate was obtained. Sherlock (1946) drew attention to the difficulty of obtaining a precipitate when the amount of hippuric acid excreted was very meagre and 17.2 per cent of her cases did not show any precipitate at all. It appears probable that a higher percentage of our patients had pronounced hepatic damage, thus failing to excrete an amount that would give a precipitate. In Graph 1 the excretion in these cases has been calculated from the volume of the urine since a fixed proportion of hippuric acid, 0.33 per cent in Quick's method (*loc. cit.*) and 0.1 per cent in the Weichselbaum *et al.* (*loc. cit.*) method, always remains in solution and is not precipitated. The maximum that could be present has been taken for the Graphs and the Tables.

It is well known that disturbances of renal function may affect the excretion of hippuric acid and thus interfere with the test. The renal factor has thus to be eliminated before a lowering of excretion of hippuric acid can be regarded as an evidence of hepatic dysfunction. The examination of the urine and the blood did not show any renal disease in any of our cases and following Sherlock's (*loc. cit.*) suggestion we did the urea-clearance test simultaneously with the hippuric-

acid test in 10 cases. The renal efficiency was normal, i.e. between 75 and 125 per cent, in all cases except one and even in this case the amount of hippuric acid excreted was too low for the degree of renal inefficiency revealed by the urea-clearance test (*see* Protocol I).

Prothrombin time and the prothrombin index.—The prothrombin time was estimated according to the method described by Fullerton (1940). Napier and Das Gupta (1941) found that the normal figure for Indians was between 15 and 25 seconds. The prothrombin index per cent, i.e. $\frac{\text{normal clotting time}}{\text{observed clotting time}} \times 100$, has been calculated in the present series taking 20 seconds as the normal clotting time.

The results and their evaluation.—An analysis of the results obtained in a series of 53 cases is given in Tables III and III-a :—

TABLE III.

Prothrombin time in seconds.	Number of cases.
16 to 20 ...	9
21 to 25 ...	17
26 to 30 ...	11
31 to 35 ...	10
36 to 40 ...	3
41 to 45 ...	1
46 to 50 ...	2

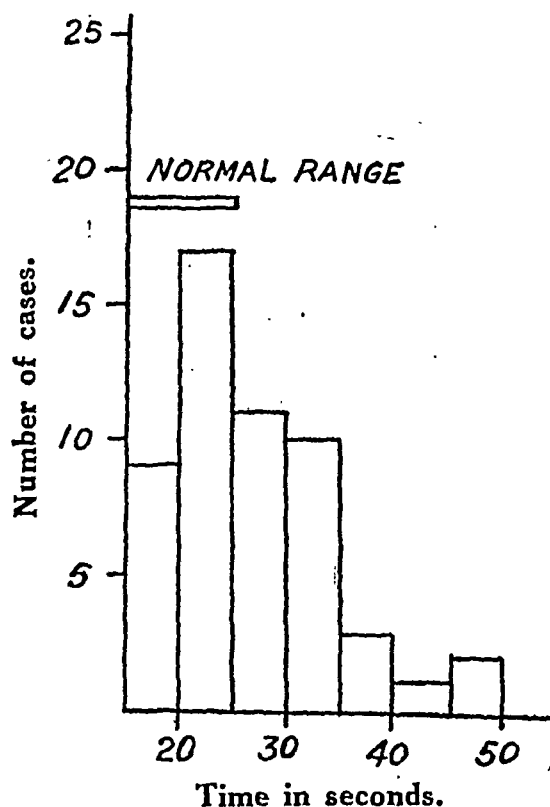
Mean and standard deviation 27.4 ± 7.6 seconds.

Taking 25 seconds as the upper limit of normal, it is found that 27 out of 53 cases, i.e. about 51 per cent of the cases, had prolonged prothrombin time (*vide* Graph 2 and Protocol II).

TABLE III-a.

Prothrombin index per cent.	Number of cases.
31 to 50 ...	4
51 to 70 ...	17
71 to 90 ...	15
91 to 110 ...	12
111 to 130 ...	5

GRAPH 2.



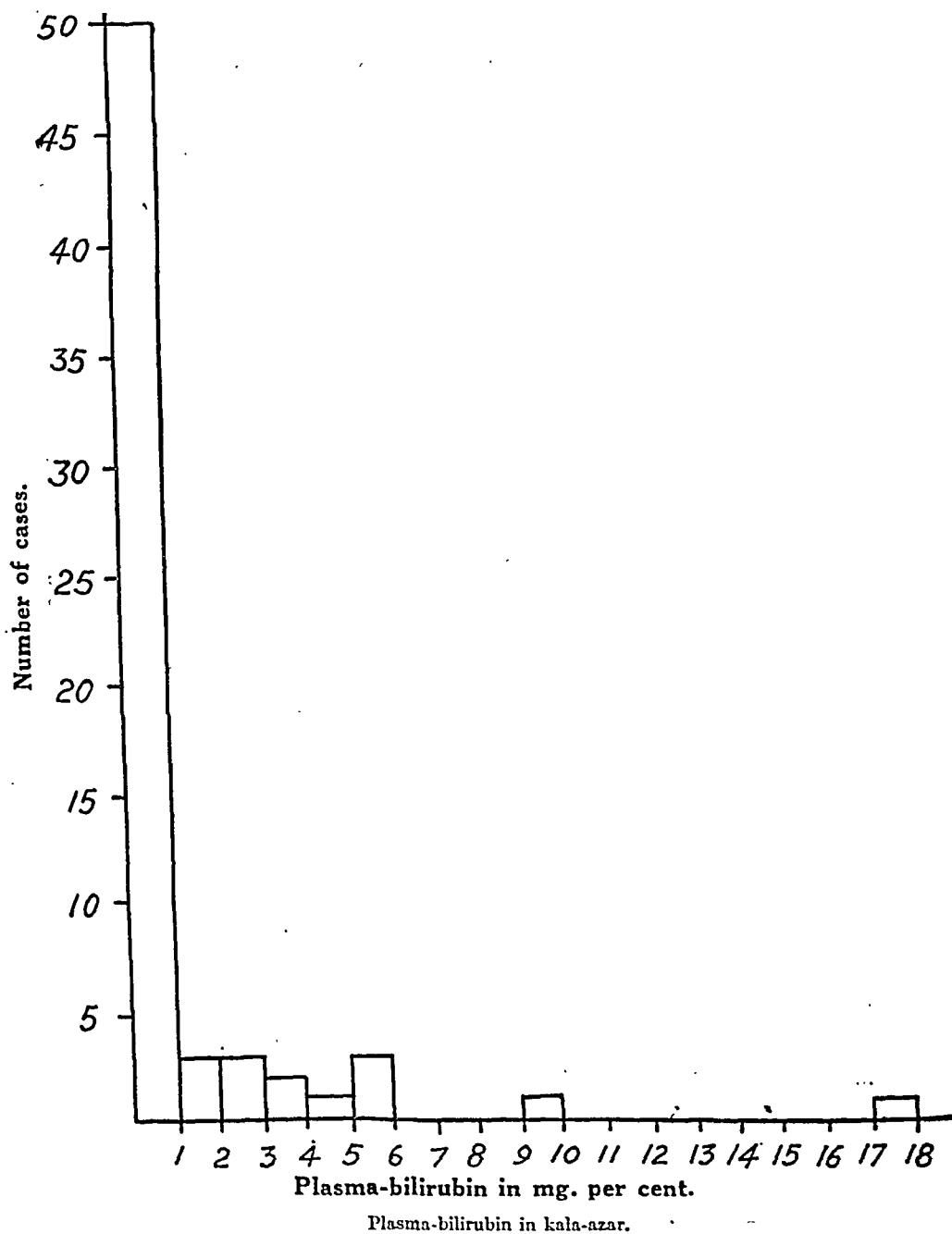
Prothrombin time with Russell's viper venom
in kala-azar (Fullerton's method).

The normal prothrombin index is regarded as lying between 90 and 110 per cent (Illingworth, 1939). Thus, in 36 out of 53 cases, i.e. 67.9 per cent of the cases, there was prothrombin deficiency. Further, on correlating the prothrombin index with the type of cases of kala-azar it has been found that 5 out of 13 early cases (38.5 per cent) and 31 out of the 40 moderate and well-developed cases (77.5 per cent) showed prothrombin deficiency. (Early case = splenic enlargement less than 2"; moderate = spleen between 2" and 4", and well-developed case = splenic enlargement more than 4" below the costal margin.)

The van den Bergh test and the bilirubin content of the plasma.—The qualitative and the quantitative van den Bergh test was performed according to the technique described by Napier and Das Gupta (*loc. cit.*) in 64 cases. The reactions obtained were as follows: Immediate direct reaction 4 cases, biphasic reaction 7 cases, delayed direct reaction 3 cases, indirect positive reaction 39 cases, and negative indirect reaction 11 cases (*vide* Graph 3. and Protocol II).

The plasma-bilirubin content of the entire series is shown in Graph 3. It will be seen that out of the 64 cases 14 showed hyperbilirubinæmia (21.9 per cent), taking 1 mg. per cent as the maximum normal figure for Indians according to

GRAPH 3.



Napier and Das Gupta (*loc. cit.*). The percentage in an unselected series of cases would perhaps be somewhat lower, because usually the more seriously ill patients are admitted into the hospital.

The serum-colloidal gold reaction.—This test was done according to the method of Maclagan (1944). In a number of cases phosphate buffer was used instead of the barbitone buffer. The results with the two buffers did not show any difference. The qualitative method was followed in all cases; and in 6 cases the quantitative method was followed as well. The results were read as follows: 5—complete precipitation, supernatant fluid colourless; 4—pale-grey blue; 3—blue; 2—reddish violet; 1—bluish red; and 0—unchanged red. The results in 104 cases are shown in Table IV and Graph 4:—

TABLE IV.

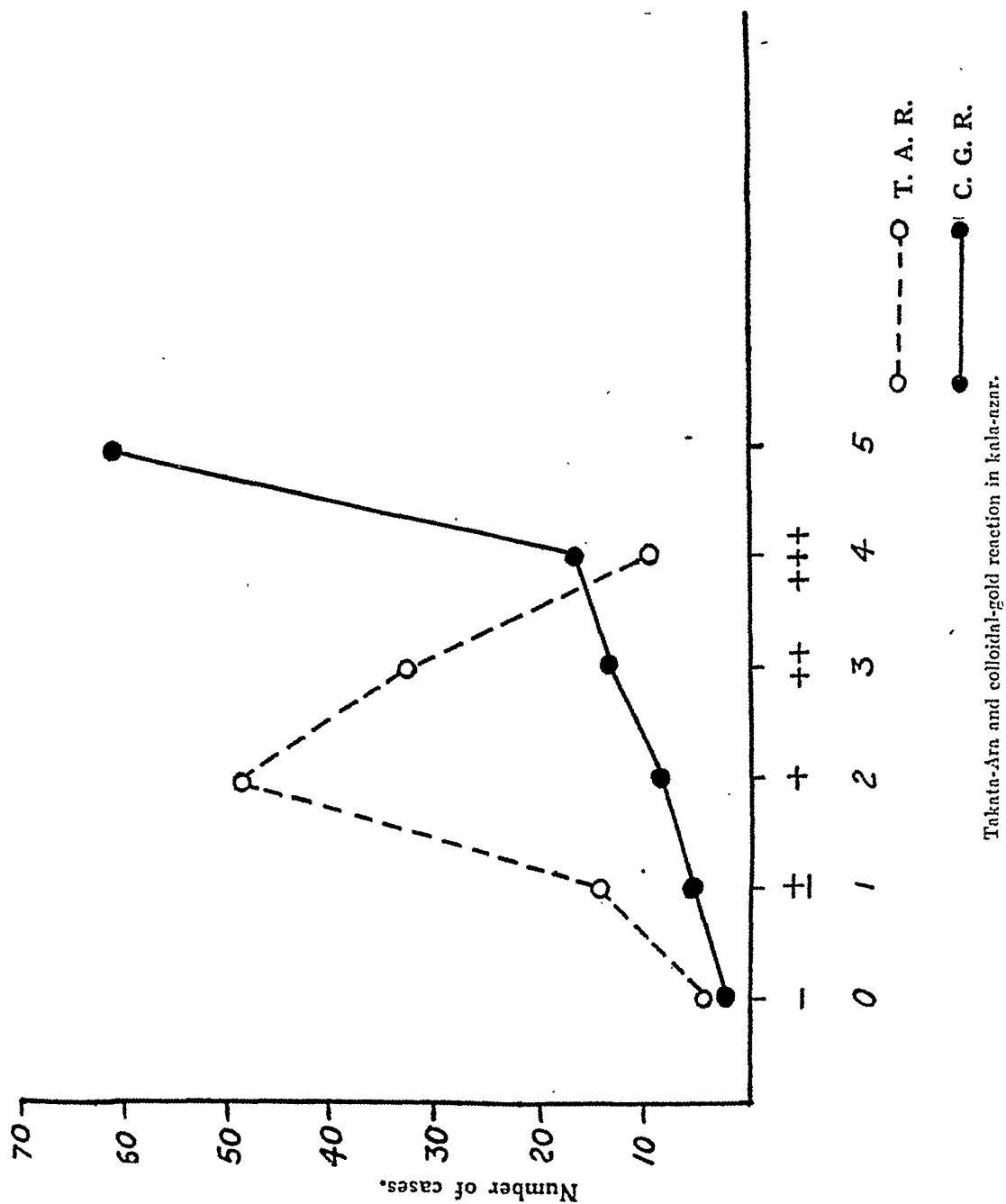
Reaction	5	4	3	2	1	0
Number of cases	...	60	16	13	8	5	2
Percentage	...	57·7	15·4	12·5	7·7	4·8	1·9

If the reactions 5 to 1 are regarded as positive, the percentage of positive reactions in kala-azar works out to be 98·1. The results of the quantitative tests are given below:—

Case.	Reaction.	REMARKS.
A ...	443210	
B ...	55555554100*	
C ...	555555541000*	
D ...	554310	On admission; vdB—biphasic, 3 mg. per cent.
	543200	After treatment; vdB—indirect, 1·25 mg. per cent.
E ...	555555	Before treatment; vdB—biphasic, 18 mg. per cent.
	555543	After 5 injections; vdB—indirect, 6·6 mg. per cent.
F ...	555555	A well-developed case, no jaundice.

* In these cases the test was done with serum dilutions from 1/60, 1/120, 1/240..... to 1/122,890 using 12 tubes. This had to be done in order to find the end-point of the reaction. Maclagan (1944, 1944a) reported that positive reactions are rare after the 4th tube; but we obtained positive reactions up to the 10th tube in kala-azar.

GRAPH 4.



It will be seen that paretic type of curve is obtained in kala-azar, and that too even in the absence of jaundice.

On analysis of the clinical data relating to this series of cases it was found that the reaction was positive in 94.1 per cent of the early cases, 96.8 per cent of the moderate cases and 100 per cent of the well-developed cases. A 5 or 4 reaction was obtained in 52.9 per cent of the early cases, 71.4 per cent of the moderate cases and 73.8 per cent of the well-developed cases.

The Takata-Ara test.—This test was carried out according to Crane's modification of Jezler's method (Crane, 1934). The criterion of positive reaction was the same as that of Kolmer and Boerner (1938). The results are given in Table V (*vide* also Graph 4) :—

TABLE V.

Reaction.	Number of cases.	Percentage.
Strongly positive ...	9	8.4
Moderately positive ...	32	29.9
Positive ...	48	44.9
Doubtful ...	14	13.1
Negative ...	4	3.7
TOTAL ...	107	100.0

On analysing the results according to the course of the disease, it has been found that in 75 per cent of the early cases, 82.9 per cent of the moderate cases and 89.4 per cent of the well-developed cases a positive reaction was obtained. In 5 cases the test was repeated after treatment. It was found that there was a tendency of the reaction to be less positive or doubtful, i.e. a shift to the right after treatment.

Serum-proteins.—The serum-proteins were estimated by the micro-Kjeldahl method. In all, 40 cases were studied, and the results are summarized in Tables VI, VII, VIII and IX, Protocol III and Graphs 5, 6, 7 and 8 :—

TABLE VI.

Total protein in g. per cent.	Number of cases.
4.1 to 5 ...	4
5.1 to 6 ...	12
6.1 to 7 ...	10
7.1 to 8 ...	11
8.1 to 9 ...	1
9.1 to 10 ...	1
10.1 to 11 ...	1

TABLE VII.

Albumin in g. per cent.	Number of cases.
1.1 to 2 ...	9
2.1 to 3 ...	18
3.1 to 4 ...	10
4.1 to 5 ...	3

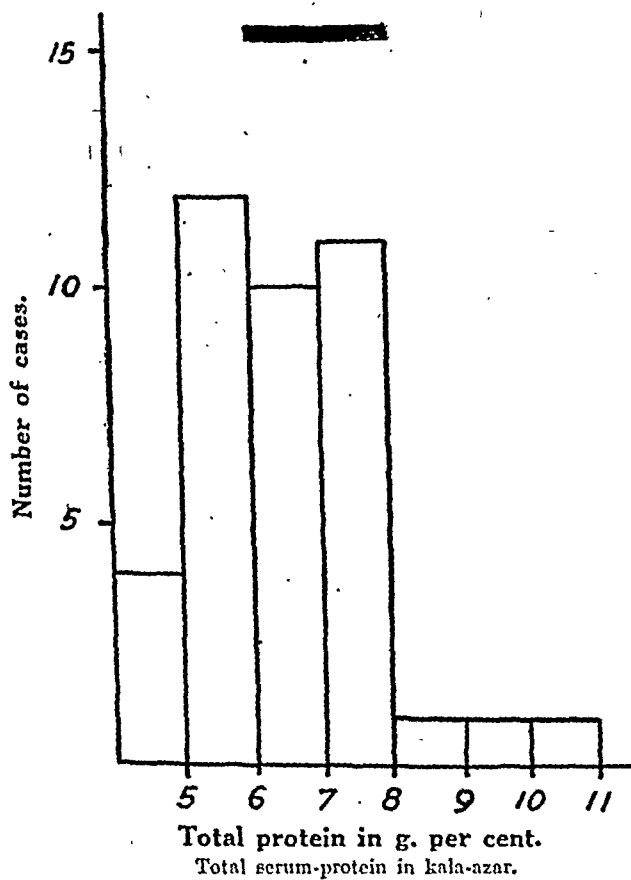
TABLE VIII.

Globulin in g. per cent.	Number of cases.
1.1 to 2	3
2.1 to 3	8
3.1 to 4	16
4.1 to 5	7
5.1 to 6	5
6.1 to 7	0
7.1 to 8	1

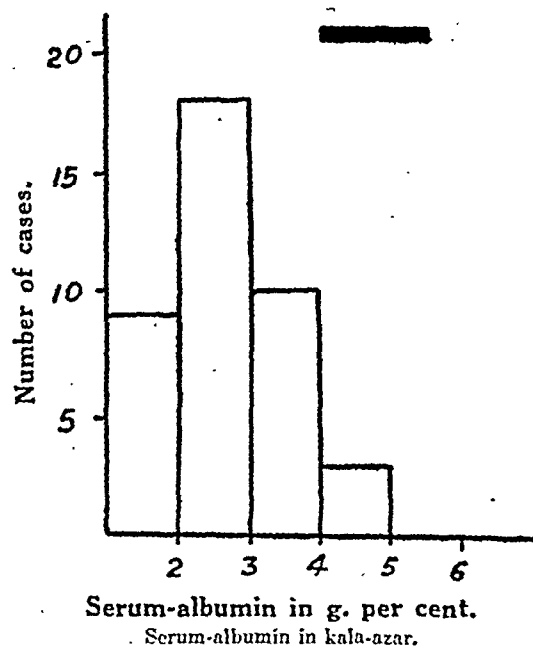
TABLE IX.

Albumin : globulin ratio.	Number of cases.
0.00 to 0.5	10
0.51 to 1.0	19
1.01 to 1.5	8
1.51 to 2.0	1
2.01 to 2.5	2

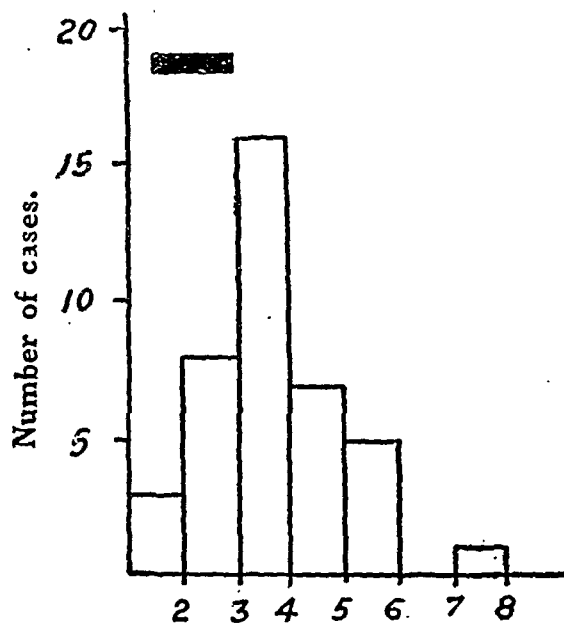
GRAPH 5.



GRAPH 6.



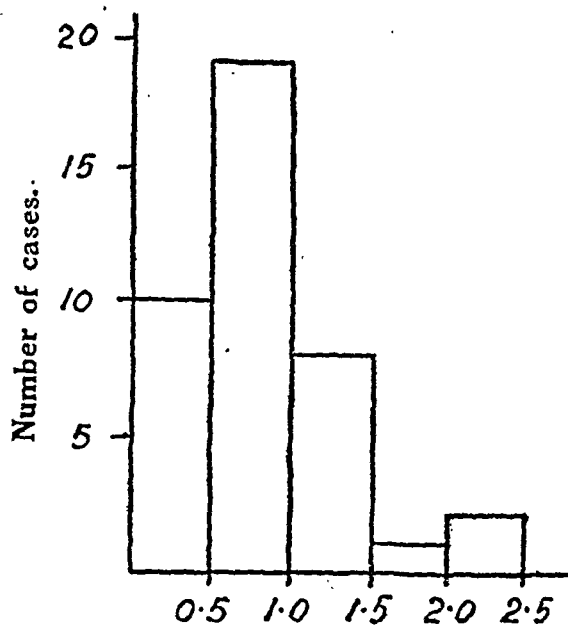
GRAPH 7.



Serum-globulin in g. per cent.

Serum-globulin in kala-azar.

GRAPH 8.



A : G ratio.

Albumin : globulin ratio in kala-azar.

TABLE X.

	Total protein.	Albumin.	Globulin.	Albumin: globulin.
Normal values, mean and s.d. (Higgins <i>et al.</i> , <i>loc. cit.</i>).	7.1	4.6	2.2	2.0
	0.38	0.29	0.36	
Mean and s.d. of the 40 cases of kala-azar	6.59	2.81	3.78	0.86
	1.25	0.88	1.20	
Average values for 12 early cases	6.3	2.8	3.5	0.94
Average values for 12 moderate cases	6.7	2.9	3.8	0.91
Average values for 16 well-developed cases	6.6	2.7	3.9	0.75

Taking 6 g. to 8 g. of total protein, 4 g. to 5.5 g. of albumin and 1.5 g. to 3 g. of globulin as the normal range, the proportion of normal and abnormal values are as shown in Table XI :—

TABLE XI.

	HIGH.		NORMAL.		LOW.	
	Number.	Per cent.	Number.	Per cent.	Number.	Per cent.
Total protein ...	3	7.5	24	60	13	32.5
Albumin ...	0	0	6	15	34	85
Globulin ...	29	72.5	10	25	1	2.5

It will be noted that hypo-albuminæmia and hyper-globulinæmia with lowering or reversal of the albumin : globulin ratio are the essential changes in the serum-proteins in kala-azar. As the disease progresses these changes become more marked. In the present series albumin content was lowered in 85 per cent of cases and globulin raised in 72.5 per cent and the albumin : globulin ratio was reduced (below 1.5) in 92.5 per cent.

On attempting to correlate the protein changes with the degree of anæmia it was found that no such correlation existed in the average cases of kala-azar with moderate degree of anæmia. In the three cases with grave anæmia, hæmoglobin below 3.6 per cent, it was, however, found that the total protein and albumin were low; the globulin was increased in one, normal in one and reduced in one. It appears that in the presence of severe anæmia, while there is a very marked reduction of the albumin fraction, the globulin is not increased to a high level. The number of cases with grave anæmia being only 3, this question cannot, however, be regarded as finally decided.

DISCUSSION.

It has been shown above with the six tests of hepatic function that reactions indicative of hepatic damage occur in a high proportion of kala-azar cases. The hippuric-acid test was positive in 71 per cent of cases, the prothrombin index was below normal in 68 per cent, there was hyper-bilirubinæmia in 22 per cent, the serum-colloidal gold reaction was positive in 98 per cent, the Takata-Ara test was positive in 83 per cent, and significant alterations of the serum-proteins was obtained in about 90 per cent of the cases (hypo-albuminæmia 85 per cent, lowering of albumin : globulin ratio 92.5 per cent). It has also been found that the frequency and the degree of positive reactions increase with the progress of the disease.

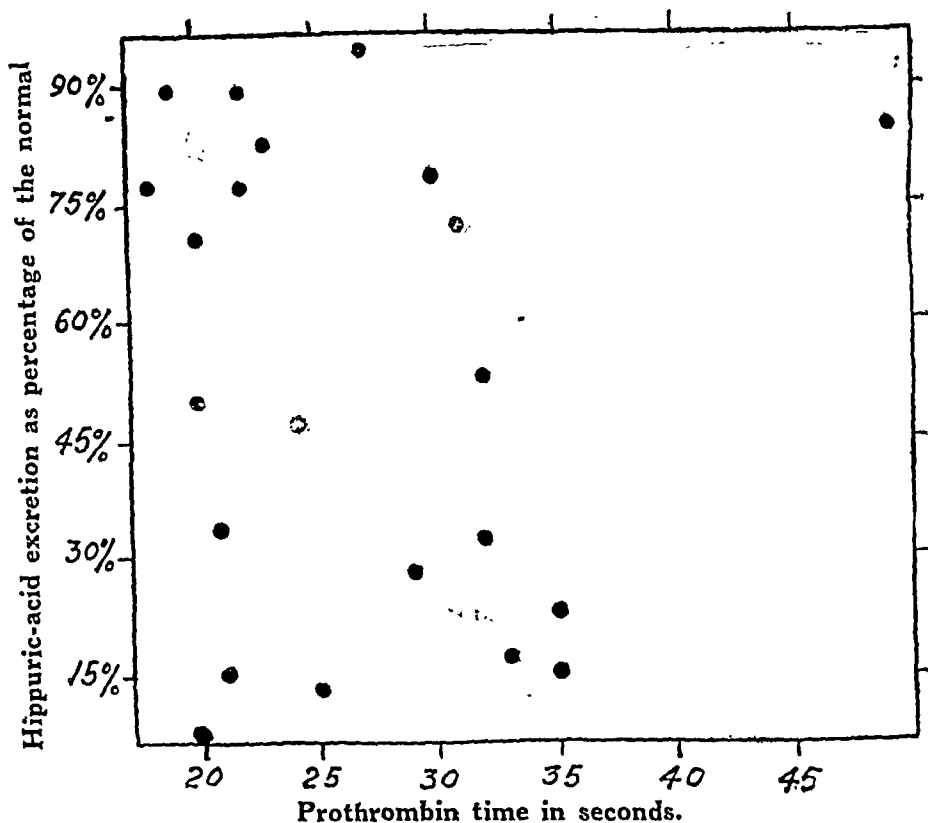
The six tests carried out indicate the status of widely different functions of the liver, and these can be placed under the following groups : (i) detoxicating

function, hippuric-acid test, (ii) synthesis of prothrombin, prothrombin-time estimation, (iii) pigmentary function, the van den Bergh test and the estimation of the bilirubin content, and (iv) the proteogenic function indicated by alteration in the serum-proteins, and the precipitation reactions such as the colloidal-gold reaction and the Takata-Ara test. The above tests give valuable indication of hepatic function and in minor degrees of hepatic damage a positive reaction may be obtained with some of the tests, while others may give a negative reaction; thus indicating the impairment of some of the functions of the liver, while others are retained at normal (Snell and Magath, 1938). When all the tests show a positive reaction in a high proportion of cases, it can be safely concluded that there has been a general derangement of the functions of the liver.

The hippuric-acid synthesis test is an established method of assessing the liver function and the intravenous administration of the sodium-benzoate solution eliminates an uncertain factor of absorption from the intestines. When (a salt of) benzoic acid is administered, it is conjugated with glycine and excreted in the urine in the form of hippuric acid. Quick (1936) has shown that a diminished excretion of hippuric acid is due to impaired capacity of the liver to synthesize glycine and defect in the enzymatic mechanism that combines glycine with benzoic acid. In man the conjugating enzyme is present mainly in the liver though a small proportion is present in the kidneys as well. We have shown that the renal function was not at fault in our series of cases and even after taking into consideration the normal variations that occur with body-weight there remain 62 per cent of cases where the lowered excretion of hippuric acid can only be explained on the basis of hepatic damage.

The prothrombin time is an index of the prothrombin content of blood. Vitamin K is essential for the synthesis of prothrombin. When the parenchyma cells of the liver do not function properly this synthesis is hampered, and the prothrombin content of the blood falls and hæmorrhages may occur. The lowering of the prothrombin content of blood may be due either to the shortage of vitamin K or to improper utilization of the vitamin by the liver. The shortage of vitamin K in the system may be caused by: (i) deficiency of the vitamin in the diet, (ii) alteration of the bacterial flora of the intestines, and (iii) faulty absorption either due to the absence of bile in the intestines or unhealthy condition of the mucous membrane. In view of the fact that these factors were not operating in most of our cases and that other tests have shown the presence of hepatic dysfunction in kala-azar, the most likely reason of the subnormal prothrombin index in 68 per cent of cases is defective hepatic function. The question whether there was a lack of vitamin K or actually the synthesis of the prothrombin in the liver was at fault can be determined only by observing the effect of parenteral administration of vitamin K on the prothrombin time. This work will be included when the studies on the biochemistry of kala-azar are further extended. It will be noted that the lowered prothrombin level and defective excretion of hippuric acid occurred in approximately 60 to 70 per cent of our cases of kala-azar. We have not, however, been able to find any correlation between the prothrombin time and the amount of hippuric-acid excretion (*see* Graph 9) and the plasma-bilirubin level. It is, however, well known that the different functions of the liver can be differently affected in hepatic disease.

GRAPH 9.



Correlation chart between hippuric-acid test and prothrombin time.

The value of the qualitative van den Bergh reaction in the differentiation of jaundice has been questioned by recent workers. Maclagan (1944, 1944a) and others have advanced substitutes which are said to give a better indication of the type of jaundice. The indirect van den Bergh test or estimation of bilirubin is, however, a valuable index of pigmentary function of the liver and has found a place in the list of the tests of hepatic function recommended by Higgins *et al.* (*loc. cit.*). It was shown by McMaster and Rous (1921) that where 5 per cent of the liver tissue is functioning jaundice may be prevented. A retention jaundice is usually produced by defective liver function with excessive hæmolysis, but when the hepatic function is more gravely affected, the normal amounts of bilirubin may not be excreted and jaundice may occur. In the present series of cases all the van den Bergh reactions can be explained on the basis of hepatic dysfunction. Three stages of dysfunction can be distinguished: (i) bile pigments are normally excreted in spite of some hepatic damage being indicated by other tests; the bilirubin content of plasma is normal, (ii) the liver cells are incapable of transferring the entire amount of bilirubin to the bile passages, there is a slight or moderate

frequency and the degree of the positive reactions increase with the progress of the disease. The mechanism of causation of the positive reactions with the above hepatic function tests in kala-azar have been discussed and it has been shown that certain clinical features of kala-azar could be explained on the basis of hepatic dysfunction.

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PROTOCOL

Results of the intravenous hippuric-

Serial number.	Case number.	Age.	Sex.	Type of case.	Body-weight in lb.	Vol. of urine in c.c.	Calculated normal excretion of hippuric acid in g.	Hippuric acid excreted in g.
1	80	34	M	Mod	79	68	0·87	0·73
2	82	28	M	E	102	148	1·02	0·15 or less
3	81	35	M	E	100	70·5	1·01	0·32
4	88	18	F	W	68	83	0·79	0·13
5	89	17	M	W	56	34	0·71	Trace
6	90	28	M	Mod	86	142	0·91	0·14 or less
7	85	23	M	E	80	145·5	0·87	0·15 or less
8	84	14	F	W	62	120	0·75	0·12 or less
9	73	25	M	W	107	60	1·05	0·35
10	74	16	M	Mod	73	57	0·83	0·83
11	77	14	F	E	?	123	...	0·57
12	102	20	M	Mod	101	58	1·01	0·40
13	103	16	M	W	89	112	0·93	0·75
14	56	25	M	E	69	45	0·80	0·05 or less
15	79	30	F	W	58	86	0·73	0·65
16	72	16	M	W	78	88	0·86	0·40
17	47	45	M	E	103	130	1·03	0·43 or less
18	46	22	M	E	103	...	1·03	0·65
19	11	22	M	Mod	85	37	0·91	0·12 or less
20	12	11	M	W	41	42	0·61	0·50
21	14	12	M	W	64	108	0·77	0·36 or less
22	15	26	M	W	80	72	0·87	0·50
23	16	40	M	E	89	40	0·93	0·65
24	17	45	M	Mod	119	95	1·13	0·87

N.B.—Results of the simultaneous urea-clearance test are also shown in 10 cases. Type of clearance.

I.

acid test in 55 cases of kala-azar.

Hippuric acid excreted expressed as benzoic acid in g.	Percentage excretion of the calculated normal amount.	Percentage excretion of the amount administered.	Blood urea mg. per cent.	Urine urea g. per cent.	Urea clearance in c.c.	Renal efficiency, per cent.
0.50	84	33	32.0	2.0	Cs66.2	122
0.10 or less	15 or less	7 or less	34.3	0.8	Cm57.6	76.6
0.22	32	15	30.0	102	Cs43.2	79.9
0.09	16	6	26.8	1.2	Cs58.9	108.8
Trace	26.8	1.45	Cs40.5	75
0.10 or less	15 or less	7 or less	30.0	0.8	Cm63.2	84
0.10 or less	17 or less	7 or less	23.6
0.08 or less	16 or less	5 or less	23.6
0.24	33	16	47.1	1.55	Cs32.9	62
0.56	100	37	32.1
0.30	...	26	29.3	0.9	Cm63.0	83.8
0.27	40	18	36.0	1.7	Cs45.8	84.7
0.51	81	34	31.0	1.1	Cs48.25	89.3
0.03 or less	6 or less	2 or less	30.0
0.44	89	29	34.3
0.27	47	18	33.0
0.29 or less	42 or less	19 or less	30.0
0.44	63	29	35.4
0.08 or less	13 or less	5 or less	34.3
0.34	82	23	27.9
0.24 or less	47 or less	16 or less	38.6
0.34	57	23	27.9
0.44	70	29	31.1
0.59	77	39	34.3

case: E=early; Mod=moderate; W=well-developed; Cm=maximum clearance; Cs=standard

PROTOCOL

Serial number.	Case number.	Age.	Sex.	Type of case.	Body-weight in lb.	Vol. of urine in c.c.	Calculated normal excretion of hippuric acid in g.	Hippuric acid excreted in g.
25	19	25	M	E	86	...	0.91	0.70
26	21	23	M	Mod	91	...	0.95	0.85
27	22	12	M	E	38	86	0.59	0.28 or less
28	23	17	M	W	112	...	1.09	0.90
29	24	10	F	Mod	49	61	0.67	0.20 or less
30	25	14	M	Mod	60	92	0.74	0.30 or less
31	100	25	M	W	72	...	0.82	0.4
32	18	18	M	Mod	83	80	0.89	0.95
33	26	10	M	W	43	46	0.63	0.15 or less
34	27	11	M	Mod	46	...	0.65	0.55
35	31	22	F	W	76	46	0.85	0.15 or less
36	32	18	F	W	74	...	0.83	0.70
37	33	14	M	E	66	...	0.78	0.75
38	35	24	M	W	97	...	0.99	0.80
39	36	50	M	E	81	...	0.88	0.20
40	43	34	M	W	119	100	1.13	0.33 or less
41	45	50	M	W	125	...	1.17	0.70
42	110	25	M	Mod	?	70	...	0.70
43	49	32	M	W	93	50	0.96	0.7
44	51	16	M	W	58	30	0.73	0.10 or less
45	53	16	M	Mod	84	...	0.90	0.62
46	55	12	M	W	48	43	0.66	0.35
47	58	28	M	Mod	80	60	0.87	0.20 or less
48	59	15	M	Mod	74	75	0.83	0.40

N.B.—Results of the simultaneous urea-clearance test are also shown in 10 cases. Type of clearance.

I—contd.

Hippuric acid excreted expressed as benzoic acid in g.	Percentage excretion of the calculated normal amount.	Percentage excretion of the amount administered.	Blood urea mg. per cent.	Urine urea g. per cent.	Urea clearance in c.c.	Renal efficiency, per cent.
0.48	77	32	42.9
0.58	89	39	30.0
0.19 or less	47 or less	13 or less	34.3
0.61	83	41	31.1
0.14 or less	30 or less	9 or less	37.5
0.20 or less	41 or less	13 or less	30.0
0.27	49	18	38.6
0.65	107	43
0.10 or less	24 or less	7 or less
0.37	85	25
0.10 or less	18 or less	7 or less
0.48	84	32
0.51	66	34
0.54	81	36
0.14	23	9
0.22 or less	29 or less	15 or less
0.48	60	32
0.48	...	32
0.51	78	34
0.07 or less	14 or less	5 or less
0.42	69	28
0.24	53	16
0.14 or less	23 or less	9 or less
0.27	48	18

case: E=early; Mod=moderate; W=well-developed; Cm=maximum clearance; Cs=standard

Serial number.	Case number.	Age.	Sex.	Type of case.	Body-weight in lb.	Vol. of urine in c.c.	Calculated normal excretion of hippuric acid in g.	Hippuric acid excreted in g.
49	69	14	M	Mod	66	55	0.78	0.65
50	108	20	M	W	117	38	1.12	0.20
51	109	19	M	W	82	50	0.89	0.65
52	34	22	F	E	58	46	0.79	0.15 or less
53	48	22	M	Mod	99	160	1.00	0.8
54	61	56	M	Mod	87	...	0.92	0.7
55	60	22	M	E	78	100	0.86	0.33 or less

N.B.—Results of the simultaneous uræa-clearance test are also shown in 10 cases. Type of clearance.

I—concl'd.

Hippuric acid excreted expressed as benzoic acid in g.	Percentage excretion of the calculated normal amount.	Percentage excretion of the amount administered.	Blood urea mg. per cent.	Urine urea g. per cent.	Urea clearance in c.c.	Renal efficiency, per cent.
0.44	83	29
0.14	18	9
0.44	73	29
0.10 or less	19 or less	7 or less
0.54	80	36
0.48	76	32
0.22 or less	38 or less	15 or less

case: E=early; Mod=moderate; W=well-developed; Cm=maximum clearance; Cs=standard

PROTOCOL

Van den Bergh reaction, plasma-bilirubin, prothrombin time,

Serial number.	Case number.	Age.	Sex.	Type of case.	Van den Bergh reaction.	Bilirubin (mg. per cent).	Prothrombin time in seconds.
1	2	16	M	W	I	0.3	36
2	3	9	F	Mod	N	0.0	49
3	4	42	M	Mod	N	0.0	25
4	5	12	F	W	I	0.2	27
5	6	12	M	W	I	0.4	30
6	7	30	M	W	I	0.2	22
7	8	32	M	E	N	0.0	18
8	9	26	M	E	I	0.6	22
9	10	30	M	E	I	0.3	21
10	11	22	M	Mod	Im.D	10.0	25
11	12	11	M	W	I	0.2	23
12	13	8	F	Mod	N	0.0	16
13	16	40	M	E	I	0.2	20
14	17	45	M	Mod	I	0.4	22
15	18	18	M	Mod	I	0.2	27
16	19	25	M	E	N	0.0	18
17	20	17	F	Mod.W	I	0.4	40
18	21	23	M	Mod	I	0.3	19
19	22	12	M	E	I	0.4	...
20	49	32	M	W	I	0.3	30
21	50	15	F	E	DD	1.5	34
22	53	16	M	Mod	N	0.0	31
23	63	21	M	E	I	0.6	24

N.B.—The prothrombin index is calculated taking 20 seconds as the normal prothrombin
 DD=delayed direct; I=indirect; N=negative. Type of case: E=early; Mod=

II.

platelet count, bleeding time and coagulation time in 67 cases.

Prothrombin index per cent.	Platelet per c.mm.	Bleeding time in minutes (Duke's method).	Coagulation time in minutes (capillary tube method).	History of hæmorrhages.
56	120,800	2.0	2.5	Epistaxis and bleeding from gums.
41	106,500	4.5	2.0	Epistaxis.
80	247,800	4.0	3.5	Bleeding from gums.
74	152,300	5.0	5.5	<i>Nil.</i>
67	157,000	1.5	3.0	<i>Nil.</i>
91	252,000	2.5	3.0	Epistaxis and bleeding from gums.
111	222,700	2.0	3.5	<i>Nil.</i>
91	308,700	1.5	3.5	<i>Nil.</i>
95	237,500	1.0	3.5	<i>Nil.</i>
80	338,200	1.0	4.0	Epistaxis and bleeding from gums.
87	327,600	2.0	3.5	<i>Nil.</i>
125	108,500	3.0	4.0	<i>Nil.</i>
100	...	2.5	5.0	<i>Nil.</i>
91	80,000	6.5	3.0	Bleeding from gums.
74	152,300	3.5	4.5	<i>Nil.</i>
111	152,400	2.0	3.5	Bleeding from gums.
50	63,800	3.5	4.5	<i>Nil.</i>
105	133,500	1.0	3.5	<i>Nil.</i>
...	72,500	4.0	6.0	<i>Nil.</i>
67	64,100	4.0	3.0	Epistaxis and bleeding from gums.
59	74,200	2.5	5.0	Bleeding from gums.
65	170,500	Bleeding from gums.
83	81,700	3.0	4.0	<i>Nil.</i>

time for Indians. Van den Bergh reaction: Im.D=immediate direct; B=biphasic; moderate; W=well-developed.

Serial number.	Case number.	Age.	Sex.	Type of case.	Van den Bergh reaction.	Bilirubin (mg. per cent).	Prothrombin time in seconds.
24	64	22	M	W	N	0.0	24
25	67	11	M	W	N	0.0	18
26	68	16	M	Mod	I	0.4	38
27	69	14	M	Mod	I	0.2	31
28	71	10	M	Mod	I	0.6	...
29	80	34	M	Mod	B	1.75	49
30	81	35	M	E	I	0.3	32
31	82	28	M	E	I	0.3	35
32	14	12	M	W	I	2.5	24
33	15	26	M	W	I	0.8	20
34	41	30	M	Mod	I	0.4	34
35	42	30	M	W	N	0.0	30
36	55	12	M	W	I	0.2	32
37	56	25	M	E	I	0.3	20
38	57	30	M	Mod	I	0.2	28
39	58	28	M	Mod	B	0.8	35
40	60	22	M	E	DD	3.0	29
41	62	20	F	W	I	1.0	28
42	70	13	M	W	N	0.0	23
43	73	25	M	W	I	0.2	21
44	76	21	M	E	I	0.5	18
45	77	14	F	E	I	0.8	23
46	79	30	F	W	N	0.0	22
47	85	23	M	E	DD	3.0	33
48	90	28	M	Mod	21

N.B.—The prothrombin index is calculated taking 20 seconds as the normal prothrombin
DD=delayed direct; I=indirect; N=negative. Type of case: E=early; Mod=

II—contd.

Prothrombin index per cent.	Platelet per c.mm.	Bleeding time in minutes (Duke's method).	Coagulation time in minutes (capillary tube method).	History of hæmorrhages.
83	144,500	3.0	2.0	Bleeding from gums.
111	101,500	1.0	2.0	<i>Nil.</i>
53	45,000	4.0	3.0	Epistaxis and bleeding from gums.
65	144,400	3.0	2.0	Epistaxis.
...	72,300	1.0	2.0	Epistaxis and bleeding from gums.
41	66,500	13.0	3.5	Epistaxis and bleeding from gums.
62	145,600	4.0	1.5	<i>Nil.</i>
57	150,000	3.0	2.0	Bleeding from gums.
83	<i>Nil.</i>
100	Epistaxis and bleeding from gums.
59	Bleeding from gums.
67	Epistaxis.
62	Epistaxis and bleeding from gums.
100	Bleeding from gums.
71	Epistaxis.
57	Epistaxis and bleeding from gums.
69	Epistaxis, bleeding from gums and melæna.
71	Bleeding from gums.
87	Epistaxis.
95	Bleeding from gums.
111	Bleeding from gums.
87	<i>Nil.</i>
91	Bleeding from gums.
61	<i>Nil.</i>
95	<i>Nil.</i>

time for Indians. Van den Bergh reaction: Im.D=immediate direct; B=biphasic; moderate; W=well-developed.

PROTOCOL

Serial number.	Case number.	Age.	Sex.	Type of case.	Van den Bergh reaction.	Bilirubin (mg. per cent).	Prothrombin time in seconds.
49	94	18	F	W	45
50	1	70	M	Mod	I	0.2	30
51	95	31	M	Mod	I	0.8	31
52	112	10	F	W	B	18.0	26
53	93	28	F	E	B	4.0	21
54	100	25	M	W	25
55	111	18	M	Mod	Im.D	6.0	26
56	25	14	M	Mod	B	3.5	...
57	26	10	M	W	I	0.6	...
58	31	22	F	W	I	0.4	...
59	32	18	F	W	I	0.2	...
60	34	22	F	E	Im.D	2.0	...
61	44	32	M	Mod	I	0.3	...
62	47	45	M	E	I	0.8	...
63	72	16	M	W	I	0.4	...
64	86	10	M	E	B	6.0	...
65	98	24	M	W	I	0.4	...
66	105	6	F	W	Im.D	6.0	...
67	107	4	F	E	B	3.0	...

N.B.—The prothrombin index is calculated taking 20 seconds as the normal prothrombin.
 DD=delayed direct; I=indirect; N=negative. Type of case: E=early; Mod=

II—concl'd.

Prothrombin index per cent.	Platelet per c.mm.	Bleeding time in minutes (Duke's method).	Coagulation time in minutes (capillary tube method).	History of hæmorrhages.
44	Nil.
67	Nil.
65	Nil.
77	Bleeding from gums and petechial hæmorrhages on both the legs.
95	Nil.
80	Bleeding from gums.
77	Bleeding from gums.
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time for Indians. Van den Bergh reaction: Im.D=immediate direct; B=biphasic; moderate; W=well-developed.

PROTOCOL III.

Estimation of serum-proteins.

Serial number.	Case number.	Age.	Sex.	Type of case.	Total protein in g. per cent.	Albumin in g. per cent.	Globulin in g. per cent.	Albumin : globulin ratio.	Takata-Ara test.	Colloidal-gold reaction.
1	1	70	M	Mod	4.2	2.2	2.0	1.20	±	4
2	2	16	M	W	5.3	2.9	2.4	1.21	+	3
3	3	9	F	Mod	5.7	3.2	2.5	1.28	-	3
4	4	42	M	Mod	6.0	2.9	3.1	0.94	++	3
5	5	12	F	W	6.4	2.9	3.5	0.83	++	4
6	6	12	M	W	7.4	2.0	5.4	0.37	+++	5
7	7	30	M	W	7.4	4.0	3.4	1.17	+	ND
8	8	32	M	E	6.2	3.8	2.4	1.58	-	0
9	9	26	M	E	7.5	3.6	3.9	0.92	+	3
10	10	30	M	E	8.0	4.0	4.0	1.00	+	4
11	11	22	M	Mod	6.8	2.1	4.7	0.44	+	4
12	12	11	M	W	7.6	2.9	4.7	0.61	+	3
13	13	8	F	Mod	7.2	1.8	5.4	0.33	++	5
14	14	12	M	W	7.8	4.0	3.8	1.05	++	5
15	15	26	M	W	7.6	1.8	5.8	0.31	+++	5
16	16	40	M	E	8.4	4.2	4.2	1.00	++	4
17	17	45	M	Mod	10.3	4.7	5.6	0.83	++	5
18	19	25	M	E	6.8	1.8	5.0	0.36	++	3

19	20	17	F	W	5.7	2.5	3.2	0.78	+++	4
20	21	23	M	Mod	7.9	2.9	5.0	0.58	+++	2
21	22	12	M	E	7.2	2.1	5.1	0.41	+++	5
22	23	17	M	W	6.8	3.6	3.2	1.12	++	1
23	24	10	F	Mod	7.6	3.8	3.8	1.00	+	5
24	25	14	M	Mod	5.7	2.2	3.5	0.63	++	4
25	26	12	M	W	5.7	3.1	2.6	1.19	±	1
26	27	30	M	Mod	6.8	2.3	4.5	0.51	+	2
27	28	30	M	W	5.8	1.8	4.0	0.45	+	4
28	29	20	M	E	6.5	3.6	2.9	1.24	+	ND
29	30	45	M	E	5.6	2.6	3.0	0.86	±	3
30	31	32	M	W	5.8	2.1	3.7	0.56	+	0
31	32	30	M	W	5.9	2.5	3.4	0.73	++	2
32	33	25	M	Mod	6.8	4.8	2.0	2.40	±	3
33	34	25	M	E	4.2	3.0	1.2	2.50	±	1
34	35	22	M	E	4.5	1.2	3.3	0.36	+	3
35	36	21	M	W	9.2	1.9	7.3	0.26	+	5
36	37	11	M	W	6.2	2.2	4.0	0.55	ND	ND
37	38	16	M	W	5.7	2.7	3.0	0.90	+	5
38	39	18	M	Mod	6.5	3.0	3.5	0.85	++	5
39	40	26	M	E	4.8	2.0	2.8	0.71	ND	ND
40		23	M	E	6.0	1.8	4.2	0.42	+	5

Type of case: E=early; Mod=moderate; W=well-developed; ND=not done.

Clinical and biochemical findings of the

Serial number.	Case number.	Age.	Sex.	Size of the spleen below the costal margin.	Type of case.	Duration of illness (in months).	Duration of jaundice (in months).	Anorexia.	Tongue.	Colour of stools.	Hæmorrhages.
1	50	15	F	2"	E	1½	?	+	Glazed	Yellow	Bleeding gums, pet. hæm.
2	80	34	M	3"	Mod	24	?	0	Clean	"	Epist., bleeding gums.
3	34	22	F	2"	E	12	?	+	"	White	Epist., bleeding gums.
4	14	12	M	5½"	W	12	?	+	"	Yellow	Nil.
5	107	4	M	2"	E	?	?	0	"	White	Nil.
6	60	22	M	2"	E	8	1½	+	Coated	"	Melæna, epist., bleeding gums.

N.B.—Abbreviations: ND = not done. Type of case: E = early; Mod = moderate; W = well-developed. Im.D = immediate direct; B = biphasic; DD = delayed direct; I = indirect. TAR = Takata-Ara. Mio = microcytic. Other findings: Cholesterol—The figures indicate the total cholesterol content.

IV.

14 cases of kala-azar with jaundice.

LIVER.				Van den Bergh reaction.	Plasma-bilirubin, mg. per cent.	TAR.	CGR.	Prothrombin index, per cent.	Type of anaemia.	Other findings.
Size.	Consistency.	Margin.	Tender nose.							
1½"	Soft	Normal	+	DD	1.5	++	5	59	Mac	Cholesterol—60 mg. per cent. Patient was gravely ill with oedema, anaemia and extensive cancerum oris. Died in the hospital.
2"	Firm	Sharp	+	B	1.75	+	5	41	Norm	Patient had ascites with congested abdominal veins. Hipp.-acid test—84 per cent of normal. Cured.
2½"	"	"	+	Im.D	2.0	++	3	ND	Mac	Case of kala-azar with cirrhosis of liver. Four pints of ascitic fluid withdrawn. Ascitic fluid—protein 0.7 per cent, TAR strongly positive, CGR —ve. Hipp.-acid test —19 per cent (or less) of normal. Van den Bergh reaction (after treatment) I, 0.6 mg. per cent. Cured.
1½"	Soft	Normal	+	I	2.5	++	5	83	Mac	Hipp.-acid test—47 per cent (or less) of normal. Blood cholesterol—130 mg. per cent, TP 7.8 per cent, A 4 per cent, G 3.8 per cent, AG 1.05. Cured.
3"	Firm	Sharp	+	B	3.0	++	5	ND	Norm	Urine—urobilin ++, bile pigments +, bile salts nil. After treatment: Van den Bergh reaction I, 1.25 mg. per cent, TAR +, CGR still +ve (5). Stools—yellow. Cured.
2½"	"	"	+	DD	3.0	+	3	69	Mac	Hipp.-acid test—38 per cent (or less) of normal. Cholesterol—70 mg. per cent, TP 4.5 per cent, A 1.2 per cent, G 3.3 per cent, AG 0.36. Cured.

Hæmorrhages: Epist. = epistaxis; Pet. hæm. = petechial hæmorrhage. *Van den Bergh reaction:* reaction; CGR = colloidal-gold reaction. *Type of anaemia:* Mac = macrocytic; Norm = normocyctic; of blood. TP = total protein; A = albumin; G = globulin.

Serial number.	Case number.	Age.	Sex.	Size of the spleen below the costal margin.	Type of case.	Duration of illness (in months).	Duration of jaundice (in months).	Anorexia.	Tongue.	Colour of stools.	Hæmorrhages.
7	25	14	M	3"	Mod	3	?	+	Clean	White	Nil.
8	93	28	F	2"	E	2	25/30	+	Slightly coated.	"	Nil.
9	85	23	M	2"	E	21/30	14/30	0	Clean	"	Nil.
10	86	10	M	1½"	E	1	1	+	Slightly coated.	"	Melena
11	105	6	F	4"	W	5	?	0	Clean	"	Bleeding gums.
12	111	18	M	3"	Mod	3	15/30	+	Slightly coated.	Yellow	Bleeding gums.
13	11	22	M	3"	Mod	2	15/30	+	Coated	Pale yellow, previously white.	Epist., bleeding gums.
14	112	10	F	6"	W	18	2	+	Slightly coated.	White	Petechiæ on both legs, bleeding gums.

N.B.—Abbreviations: ND = not done. Type of case: E = early; Mod = moderate; W = well-developed. Im.D = immediate direct; B = biphasic; DD = delayed direct; I = indirect. TAR = Takata-Ara Mic = microcytic. Other findings: Cholesterol—The figures indicate the total cholesterol content

IV—concl'd.

LIVER.				Van den Bergh reaction.	Plasma bilirubin, mg. per cent.			Prothrombin index, per cent.	Type of anaemia.	Other findings.
Size.	Consistency.	Margin.	Tender nose.							
6"	Firm	Sharp	+	B	3.5	++	4	ND	Norm	Hipp.-acid test—41 per cent (or less) of normal. Cholesterol—85 mg. per cent, TP 5.7 per cent, A 2.2 per cent, G 3.5 per cent, AG 0.63. Van den Bergh reaction I, 1.75 mg. per cent (after treatment). Cured.
2"	Soft	Rounded	+	B	4.0	ND	ND	95	Norm	Patient had two relapses. Jaundice developed during the 3rd attack. Cured.
2"	"	"	+	DD	5.0	+	5	61	Mic	Hipp.-acid test—17 per cent (or less) of normal. Cholesterol—125 mg. per cent, TP 6 per cent, A 1.8 per cent, G 4.2 per cent, AG 0.42. Urine—urobilin ++, bile pigments +, bile salts +. Cured.
2"	Firm	Sharp	0	B	6.0	++	5	ND	ND	Patient had ascites with general anasarca. Cured.
2½"	"	"	+	Im.D	6.0	++	5	ND	ND	Cured.
3½"	"	"	+	Im.D	6.0	ND	ND	77	Norm	Appetite was good before but was lost when jaundice appeared. Cured.
2"	"	"	+	Im.D	10.0	+	4	80	Norm	Hipp.-acid test—13 per cent (or less) of normal. Cholesterol—145 mg. per cent, TP 6.8 per cent, A 2.1 per cent, G 4.7 per cent, AG 0.41. Agglutination against <i>L. icterohæmorrhagica</i> —neg. Van den Bergh reaction (after treatment) DD 3.5 mg. per cent, CGR 3, TAR +ve. Cured.
2½"	"	"	+	B	18.0	+	5	77	ND	Patient had ascites and oedema. Van den Bergh reaction I (after 5 injections of aminostilburea) 0.6 per cent. Stools—yellow, liver as before, no fluid in abdomen, no oedema. TAR ±. Cured.

Hæmorrhages: Epist. = epistaxis; Pet. hæm. = Petechial hæmorrhage. Van den Bergh reaction; CGR = colloidal-gold reaction. Type of anaemia: Mac = macrocytic; Norm = normocytic; of blood. TP = total protein; A = albumin; G = globulin.

ON THE NATURE OF PENICILLIN BACTERIOSTASIS.

Part III.

THE INFLUENCE OF MAGNESIUM RIBO-NUCLEATE ON THE NATURE OF PENICILLIN INHIBITION OF GRAM-NEGATIVE ORGANISMS.

BY

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IN our earlier publications (Pandalai and George, 1947; George and Pandalai, 1948, 1948a) it has been shown that nucleic acids possess the property of (i) antagonizing the inhibitory action of penicillin, and (ii) they are able to reverse the inhibition already brought about in the case of organisms due to contact with penicillin. Thus, organisms rendered non-viable due to penicillin contact were found to have restored the power of growth and multiplication by added nucleic acid. It was also demonstrated that nucleic acid imparted changes in morphology and Gram-staining reaction due to contact with the drug. It was found also that nucleic acids failed to exercise any of these effects on the Gram-negative pathogens, normally insensitive to penicillin but inhibited by higher concentrations. It was argued that the Gram-staining nature of an organism had no significant relation to the sensitivity or otherwise of the organism to penicillin and that an explanation for the selectivity in action probably lay in the fact that metabolic reaction channels are different in the case of sensitive organisms on the one side and the insensitive organisms on the other. Since important clinical and therapeutic aspects centre round this problem of selectivity of the action of penicillin, it should be interesting to elucidate the various features which govern the bacteriostatic effects brought about by penicillin. In this connection the suggestion that the presence of nucleic acid in the form of magnesium ribo-nucleate is a significant factor in imparting the particular staining characteristic to an organism and that the Gram-positive organisms contain this material in the surface layer appears to have received experimental confirmation from recent findings of Henry and Stacey (1943, 1945,

1946). Indeed these workers showed that magnesium ribo-nucleate prepared from commercial sources was capable of restoring the Gram-positive quality to bacteria from which it has been removed by extracting with 2 per cent bile salt in normal saline, as much as the latter extract itself. This fact had added implications, since now it has been shown that other antibiotic substances besides penicillin are also similarly sensitive. These aspects raised the interesting point as to what influence the added magnesium ribo-nucleate would bring about in the case of Gram-negative bacteria placed in contact with penicillin. It may be expected that in the presence of added magnesium ribo-nucleate, the Gram-negative organisms which are originally insensitive may be rendered more allied in their life processes to the sensitive organisms, consequently becoming inhibited by lower concentrations of penicillin. If this was true, then it would be possible to evolve conditions in which penicillin becomes more versatile in its action and thus applicable for the therapy of infections brought about by insensitive Gram-negative organisms such as those belonging to the colon-typhoid group. The following experiments were carried out accordingly to test these postulates:

EXPERIMENTAL.

Experimental procedure was similar to the one already used in these studies and described in our previous papers (*loc. cit.*). Magnesium ribo-nucleate was prepared from pure yeast nucleic acid and used as a neutral solution of the magnesium salt of pure yeast nucleic acid. Twenty-four-hour broth-culture tubes in duplicates containing a total volume of 10 c.c. including nutrient broth and test substances added in requisite amounts were observed after 18 to 24 hours' incubation at 37°C. and the presence or absence of turbidity due to bacterial growth was noted. First the influence of small concentrations of magnesium ribo-nucleate on the penicillin action on *Esch. coli* was studied. The results are given in Table I and show that magnesium ribo-nucleate antagonizes the inhibitory action of penicillin in the case of *Esch. coli*, while it alone when present in broth does not act either as a growth-promoting or inhibiting substance.

TABLE I.

The effect of magnesium ribo-nucleate on penicillin against Esch. coli.

Penicillin alone, units per c.c.	Growth in broth.	Magnesium ribo-nucleate alone.	Growth in broth.	Magnesium ribo-nucleate plus penicillin units per c.c.	Growth in broth.	Control in broth.
12.0	+	1/2,500	+++	1/2,500 { 12 15 17.5	++ ++ ++	+++
15.0	—	1/5,000	+++	1/5,000 { 12 15 17.5	++ ++ +	
17.5	—	
20.0	—	1/20,000	+++	1/1,000 { 12 15 17.5	++ + ±	

— complete inhibition; ± scanty growth; ++ fairly good growth; +++ very good growth.

That penicillin bacteriostasis is reversible by magnesium ribo-nucleate as it was in the case of *Staph. aureus* (*loc. cit.*) by nucleic acid was made clear by adding the nucleate solution in cultures which were treated with penicillin at different intervals and incubated as usual. Results are given in Table II:—

TABLE II.

To show the reversibility of penicillin action on *Esch. coli* by the added magnesium ribo-nucleate.

Penicillin alone, units per c.c.	Growth in broth.	MAGNESIUM RIBO-NUCLEATE 1/2,500 ADDED TO BROTH.			Control in broth.
		After 3 hours.	After 6 hours.	After 24 hours.	
12.0	+	++	++	++	} ++
15.0	—	++	++	++	
17.5	—	++	++	++	
20.0	—	++	++	++	

+ growth; — inhibition; ++ fairly good growth.

The results in Tables I, II and III show that, while in the case of Gram-positive organisms the addition of nucleic acid alone can bring about the inactivation of penicillin (*loc. cit.*), the same effect is brought about in the case of Gram-negative organisms only by the addition of magnesium ribo-nucleate and not by the nucleic acid alone. That the magnesium present in the added magnesium ribo-nucleate plays an important part is confirmed by the results given in Table III:—

TABLE III.

Showing the effect of magnesium-ions supplied as $MgSO_4$ on penicillin inhibition of *Esch. coli* in the presence of added nucleic acid.

Penicillin alone, units per c.c.	Growth in broth.	MgSO ₄ alone, per cent.	Growth in broth.	Nucleic acid per cent + penicillin units per c.c.	Growth in broth.	MgSO ₄ per cent, nucleic acid + penicillin units per c.c.	Growth in broth.	Control in broth.
8.0 10.0	+	} 0.1	++	0.1 { 8 10 12 15	+ + + —	} 0.1 + $\frac{1}{1,000}$ { 8 10 12 15 20	++ ++ ++ + ±	} ++
	+							
	—							
	—							
12.0 15.0 20.0	+	} 0.2	++	0.2 { 8 10 12 15	+ + + —	} 0.2 + $\frac{1}{1,000}$ { 8 10 12 15 20	++ ++ ++ + ±	
	—							
	—							
	—							

— inhibition; + growth; ± scanty growth; ++ fairly good growth.

In these experiments magnesium-ions were supplied to the cultures as magnesium sulphate and nucleic acid was added separately.

That the magnesium-ions, apart from the ribo-nucleate moiety of magnesium ribo-nucleate, exert a pronounced influence on the action of penicillin on the growth of *Esch. coli* in the absence of added nucleic acid, is seen from the results given in Table IV :—

TABLE IV.

To show the influence of magnesium-ions added as $MgSO_4$ on the growth of *Esch. coli* in the presence and absence of penicillin.

Penicillin alone, units per c.c.	Growth in broth.	$MgSO_4$ alone, per cent.	Growth in broth.	$MgSO_4$ per cent + penicillin, units per c.c.	Growth in broth.	Control in broth.
0.0	+++	0.1	+++	6.0	++	+++
8.0	++			8.0	±	
10.0	++			10.0	—	
				12.0	—	
12.0	+	0.2	+++	6.0	++	
15.0	—			8.0	±	
17.5	—			10.0	—	
				12.0	—	

— inhibition; + growth; ± scanty growth; ++ fairly good growth; +++ very good growth.

Here the results indicate that magnesium-ions alone could accomplish a reduction in the minimum inhibiting concentrations of penicillin required by *Esch. coli*. Magnesium sulphate in different concentrations was used for the experiments with penicillin in different concentrations. A concentration of magnesium round about 0.1 per cent (as $MgSO_4$) is most active in bringing about the optimum effect, while magnesium sulphate alone was found to have no effect on the growth of the bacilli. The inhibitory effect of the combination of magnesium-ions and penicillin, therefore, does not seem to be due to changes in the pH conditions of the medium.

Similar results were obtained in the case of *B. dysenteriae* also, but not with *B. typhosus*.

In another series of experiments *Esch. coli* was allowed to grow in a nutrient broth to which had been added 0.5 per cent magnesium sulphate and subcultured into similar medium 3 or 4 times, and was used as an inoculum in order to ascertain whether such a strain having acclimatized to magnesium environment and also presumably containing some adsorbed magnesium (Dubos, 1945) would behave in any different way from the ordinary broth-culture strain. The results given in

Table V show, however, that this pre-treatment of the organisms in magnesium sulphate added broth, had adverse effect on its minimum requirements of penicillin for complete inhibition.

TABLE V.

Demonstrating the absence of effect of cell-adsorbed magnesium on the minimum inhibiting concentration of penicillin required by Esch. coli.

Penicillin + ordinary broth culture, units per c.c.	Growth in broth.	Penicillin + 0.5 per cent MgSO ₄ broth culture.	Growth in broth.	0.5 per cent MgSO ₄ broth culture.	Ordinary broth culture
8.0	++	8.0	++	} ++	++
10.0	++	10.0	++		
12.0	+	12.0	++		
15.0	—	15.0	+		
20.0	—	20.0	+		

-- inhibition; + growth; ++ fairly good growth.

This means that it is the extra-cellular magnesium that takes part in the reactions and that the cellular magnesium plays little or no part in the case of Gram-negative organisms in rendering them sensitive to lower concentrations of penicillin.

DISCUSSION.

The experimental results reported above do not help to clarify the question of the specificity found in the bacteriostatic action of penicillin. They, however, throw some light on the absence of any significance of the Gram-staining test as a deciding factor in the susceptibility or otherwise of penicillin on bacteria. There is, no doubt, considerable interest in the finding that magnesium ribo-nucleate to which has been attributed the property to impart a Gram-positive reaction to certain groups of bacteria, can bring some changes in certain Gram-negative organisms at least as much as to confer to them the power of growth from a non-viable condition to which they were transformed by penicillin contact. To this group of Gram-negative organisms belong the penicillinase producers, such as *Esch. coli* and *B. dysenteriae*, since *B. typhosus* which is not known to produce penicillinase behaves in a different way. Indeed, it has been found that magnesium ribo-nucleate stands to such Gram-negative bacteria in the same relation as nucleic acid stands to Gram-positive bacteria as far as the antagonizing effect on penicillin and the ability to reverse penicillin action are concerned. However, added magnesium ribo-nucleate in suitable concentrations was unable to render the normally insensitive organisms susceptible to lower concentrations of penicillin.

That is to say, the magnesium ribo-nucleate, in spite of the changes it is able to impart to the unsusceptible organisms, cannot render the latter more allied to the sensitive organisms judging at any rate from the minimum bacteriostatic concentration of penicillin required by them. The most striking point emerging from these results, however, is the fact that magnesium-ions themselves are able to influence the insensitive pathogens in the presence of penicillin in such a way that lower concentrations of penicillin become inhibitory to them. Magnesium has no effect when it is present in the cells as adsorbed magnesium or even as circumambient magnesium unless penicillin is also present. Under these conditions, namely, in the joint presence of magnesium-ions and penicillin, the minimum bacteriostatic concentration of penicillin for *Esch. coli* and *B. dysenteriae* go down by about 48 per cent of the amount required when penicillin is present alone. This finding may have some important significance, since it is known that the magnesium-ion acts as a co-enzyme in phosphorylation processes occurring during the catabolism of carbohydrates *in vivo*.

It may also be pointed out that the assumption that the ability to produce penicillinase may be tied up with the absence in the penicillin-resistant organisms of magnesium ribo-nucleate has also to be verified by further work on the basis of these results. It would seem that even among the Gram-negative pathogens, some, such as *Gonococci*, are very susceptible to penicillin; some, such as *Esch. coli*, *B. dysenteriae*, etc., can be made susceptible by suitable methods, while some, such as *B. typhosus*, require very high minimum inhibiting concentrations of penicillin. The similarity in the behaviour of *Esch. coli* and *B. dysenteriae* has probably some connection with their ability to produce penicillinase. It may be pointed out that *B. typhosus* is not a penicillinase producer and this may account for the different ways in which it behaves to these reagents.

Thus, it seems reasonable to suppose that in the penicillin-sensitive pathogens there is present a 'catalytic complex system' containing *inter alia* magnesium and ribo-nucleic acid and it may be assumed that only such a complete system can act, as it were, a receptor to penicillin, while the corresponding system in the non-sensitive pathogens is lacking in some component involving the functions of magnesium or ribo-nucleic acid factors which consequently cannot function as a penicillin receptor. Undoubtedly, external magnesium to some extent fulfils to render this incomplete catalytic complex system more complete, if not fully, in the insensitive pathogens with the result that the respiratory reactions of these pathogens become more allied to those in the sensitive ones. This result points to the possibility of taking advantage of the use of magnesium-penicillin mixture orally for curing the infections of the digestive tract. Preliminary experiments in mice have shown that a promising future awaits such clinical practices. These *in vivo* experiments are now in progress.

SUMMARY.

1. Magnesium ribo-nucleate allows the growth of *Esch. coli* in the presence of minimum inhibiting concentrations of penicillin.

2. *Esch. coli* which became non-viable due to contact with high doses of penicillin were rendered viable by added magnesium ribo-nucleate.

3. The important rôle played by the magnesium-ions in the bacteriostasis of these organisms by penicillin has been demonstrated. Circumambient magnesium brings down the minimum bacteriostatic concentration of penicillin by about 48 per cent.

4. The specificity of penicillin action has been attributed, on the basis of these results, to the presence in the sensitive organisms of a 'catalytic complex system' containing *inter alia* magnesium and ribo-nucleic acid factors which function as receptors to penicillin. The absence or incompleteness of this catalytic system in the insensitive pathogens accounts for their non-susceptibility to penicillin irrespective of their Gram-staining reactions.

5. Extra-cellular magnesium is able to render complete at least in part this 'catalytic complex system' in the insensitive pathogens, thus bringing the respiratory reaction pathways of these organisms more and more allied to those in the sensitive ones.

6. The possible applications of magnesium-*cum*-penicillin in clinical practice to treat infections of the intestinal tract are discussed on the basis of the results, preliminary experiments in mice having already yielded promising results.

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INVESTIGATIONS ON PLANT ANTIBIOTICS.

Part III.

PTERYGOSPERMIN—THE ANTIBACTERIAL PRINCIPLE OF THE ROOTS OF MORINGA PTERYGOSPERMA GÆRTN.

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INTRODUCTION.

DURING recent years a number of plants have been examined for their antibiotic activity (Osborn, 1943; Pederson and Fischer, 1944). In some cases the active principles have been isolated. These include the antibiotic agents from garlic (Cavallito and Bailey, 1944, 1945; Rao, 1946), from common burdock (Cavallito and Bailey, 1945a) and tomatin from the tomato plant (Irving, 1946). We have shown in a previous communication that aqueous or alcoholic extracts of several locally available plant materials possess remarkably high antibiotic activity (George, Pandalai and Venkataraman, 1947).

Of these *M. pterygosperma* exhibited exceptionally high antibiotic properties. Experiments to isolate the antibacterial principle were hence undertaken and in a previous communication (Rao and George, 1946) results of preliminary studies have been reported. This tree is deciduous, belongs to the *N. O. Moringæ* and is very common almost in every part of India. No attempt seems to have been made so far to isolate the medicinal principles of the plant even though almost all parts of the plant like the root, bark, gum, leaf, flower and seed have been extensively used for various ailments in indigenous medicine. The only product isolated from the plant is an alkaloid from its bark (Ghosh, Chopra and Dutt, 1935).

Due to the high antibiotic activity exhibited by the alcoholic extracts of the root as compared with the other parts of the plant and on account of the availability throughout the year, the root was chosen for the isolation of the antibiotic principle. In the present communication are recorded the results of investigations on the isolation, antimicrobial activity and mode of action of the antibiotic principle.

EXPERIMENTAL.

A. pterygospermin unit.—In order to facilitate the calculation of the yield at every step in isolation, we defined a unit of *ptyerygospermin* as that amount of the antibiotic which when present in 1 c.c. of sterile nutrient broth at pH 7.0 just inhibits the growth of a given strain of *S. aureus*, the amount of inoculum being 0.01 c.c. of a 24-hour broth culture. This would correspond roughly to 1 μ g. of streptomycin or 0.6 μ g. of penicillin.

Preparation.—After a series of preliminary experiments we adopted the following procedure for the isolation of *ptyerygospermin*. About 1,000 grammes of fresh roots were cut into small pieces and extracted with about 1,500 c.c. of alcohol in the cold over-night. The reddish brown alcoholic extract had an activity of about 70 units per c.c. A second extract of the root with an equal amount of alcohol showed an activity of about 10 units per c.c. To 1,500 c.c. of the first extract 10 g. of 'Norit' adsorbant was added. To an equal amount of the second extract 5 g. of the adsorbant was added. The suspensions were shaken well for about 30 minutes and filtered using celite powder. *Pterygospermin* was completely adsorbed. Of the different eluents studied light petroleum proved to be the best. The petroleum ether eluate was dried over anhydrous sodium sulphate and the solvent removed at low temperature and pressure. An oily substance with a reddish brown colour and highly piercing smell was left behind. This is the most active product so far obtained. Further purification using chromatography has been tried without any success. It has been a common observation that if the petroleum ether is not quickly removed from the eluate, the needle-shaped crystals separate, which, however, do not show any appreciable activity. *Pterygospermin* is moderately soluble in alcohol but sparingly soluble in water. In Table I the results of typical experiments on its preparation are presented :—

TABLE I.
Preparation of pterygospermin.

Number of experiment.	Weight of roots taken in g.	Alcohol used, vol. in c.c.	Activity in units of extract I.	Activity in units of extract II.	Weight of oil isolated in mg.	Percentage recovery.
1	1,000	1,500	80	10	310	69.0
2	3,000	4,500	65	5	750	71.0
3	2,800	4,300	65	5	715	71.0
4	5,350	7,500	60	10	1,250	72.0
5	1,500	2,200	70	5	425	77.0
6	4,000	5,800	65	5	900	67.0

Stability of pterygospermin.—Freshly prepared samples of *ptyergospermin* have shown very high antibacterial activity against different micro-organisms. Solutions of the antibiotic in alcohol at the laboratory temperature have retained their original activity for a period of about 2 months. Prolonged keeping of the solution, however, results in gradual loss of activity. A crystalline precipitate, having no antibacterial activity, settles down from alcoholic solutions on keeping. Heating for even an hour at the temperature of the boiling water-bath does not seem to affect the stability of *ptyergospermin*.

The effect of pH on the stability of the antibiotic was next studied since this is known to affect the activity of antibiotics like streptomycin and penicillin to a very great extent. Equal amounts of the alcoholic solutions of *ptyergospermin* and buffers of different H-ion concentrations were mixed. The emulsions so obtained were incubated for 24 hours at 37°C. At the end of this period, the antibacterial activity of the emulsions were determined by the cup-plate as well as by the serial-dilution method. The results of these experiments are brought out in Table II. It will be seen therefrom that *ptyergospermin* is more stable in the acid range, its activity falling on keeping when the reaction is even slightly alkaline. Similar experiments carried out to find out the optimum pH of its activity revealed that *ptyergospermin* best exhibits its activity at neutral reaction.

TABLE II.

Stability of pterygospermin at different H-ion concentrations.

pH of the mixture.	ACTIVITY AFTER 24 HOURS' INCUBATION.	
	Zone of inhibition diameter in mm.	Turbidity due to growth at dilution 1 in 100,000.
Alcoholic solution.	More than 40	—
5	25	—
6	22	—
7	20	—
8	15	+

— indicates no turbidity; + indicates growth.

Antibacterial spectrum.—For finding the antibacterial activity alcoholic solutions of *ptyergospermin* were diluted in nutrient broth. Due to the insoluble nature of the antibacterial agent, up to a dilution of 1 in 10,000 there was faint turbidity. Further dilution always gave clear broth enabling the assaying of the antibacterial substance. In regular assaying a solution of *ptyergospermin* in alcohol 100 mg. per c.c. was first diluted in sterile water to give dilutions of 2,000, 4,000, 8,000, etc., and 0.5 c.c. of these emulsions was added to 4.5 c.c. of nutrient

broth. These tubes were then inoculated with 0.01 c.c. of a 24-hour broth culture of the test organisms and incubated at 37°C. for 18 hours, after which the tubes were observed for growth. For acid-fast organisms the following procedure was employed: Flasks containing Long's asparagine medium, with the antibiotic in the form of an emulsion, 10 mg. per 100 c.c. of the media, were inoculated with two loopfuls of a non-pathogenic strain of *Mycobacterium tuberculosis* B.C.G. and *Mycobacterium phlei* and incubated at 37°C. No growth was observed in any of the flasks containing the extract, while in the controls the organisms grew well at the end of the two weeks. The antibacterial spectrum of *pterygospermin* for different micro-organisms tested is given in Table III:—

TABLE III.
Antibacterial spectrum of *pterygospermin*.

Test organisms.	Dilution of <i>pterygospermin</i> in broth $\times 1/1,000$								Number of units per c.c. at maximum inhibitory dilution.
	$\frac{1}{20}$	$\frac{1}{30}$	$\frac{1}{50}$	$\frac{1}{75}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{300}$	Control.	
1. <i>B. subtilis</i> ...	—	—	—	—	—	—	++	++	1
2. <i>S. aureus</i> ...	—	—	—	—	—	—	++	++	1
3. <i>B. coli</i> ...	—	—	—	+	+	+	++	++	4.5
4. <i>B. typhosus</i> ...	—	—	—	—	+	+	++	++	3.0
5. <i>B. enteritidis</i> ...	—	—	—	—	+	++	++	++	3.0
6. <i>B. acrogenes</i> ...	—	—	—	+	+	++	++	++	4.5
7. <i>B. paratyphosus</i> B.	—	—	—	+	+	++	++	++	4.5
8. <i>B. paratyphosus</i> C.	—	—	—	—	—	—	++	++	1
9. <i>B. dysenteriae</i> ...	—	—	—	—	—	—	++	++	1
10. <i>Mycobacterium phlei</i>	—	—	—	—	—	—	++	++	1
11. <i>Mycobacterium tuberculosis</i> B.C.G.	—	—	—	—	—	—	++	++	1

— indicates no growth; + indicates slight turbidity; ++ indicates growth as much as in control.

The results show that this antibiotic exhibits a very wide range of activity inhibiting the different micro-organisms including acid-fast and Gram-negative ones at fairly high dilutions.

Antifungal activity.—The experimental procedure for finding out the fungistatic activity of *ptyergospermin* was the same as that employed to find out its antibacterial properties. The tubes in this case after inoculating with the test organism were, however, kept in a slanting position and the observations for growth made after 3 to 4 days' incubation at a temperature of 25°C. The results of the experiments with a few fungi are summarized in Table IV. It is evident that *ptyergospermin* exhibits good antifungal activity also.

TABLE IV.

Antifungal properties of ptyergospermin.

Name of organism.	Medium.	DILUTION OF ANTIBIOTIC IN MEDIUM.					Number of units per c.c. at maximum inhibitory dilution.
		$\frac{1}{80,000}$	$\frac{1}{100,000}$	$\frac{1}{150,000}$	$\frac{1}{200,000}$	Control.	
1. <i>Actinomyces griseus</i> .	Nutrient broth	—	—	—	+	+	2.0
2. <i>Aspergillus ustus</i> .	Czapeck Dox medium and nutrient broth.	—	+	+	+	+	3.0
3. <i>Aspergillus fumigatus</i> .	"	—	+	+	+	+	3.0
4. <i>Penicillium notatum</i> .	"	—	+	+	+	+	3.0

— indicates no growth; + indicates growth.

Effect of ptyergospermin on SH compounds.—A number of antibiotic substances of heterogeneous chemical nature is inactivated by cysteine (Cavallito and Bailey, *loc. cit.*) and other similar compounds containing reactive sulphydryl groups. Based on these observations it has been suggested (Cavallito, *loc. cit.*) that these antibiotics act by their ability to react with the sulphydryl groups of enzyme systems. The effect of cysteine and thiosulphate on the activity of *ptyergospermin* was, therefore, studied.

Known amounts of the thiol reagents in sterile solutions at pH 6.8 were mixed with known amounts of *ptyergospermin* and the emulsions so formed incubated. At the end of definite intervals the antibiotic activity of the mixtures were assayed. Tables V and VI give the results of these studies. It will be seen that *ptyergospermin* belongs to the group of antibiotics which are not inactivated by sulphydryl reagents.

TABLE V.
Effect of cysteine on the antibacterial activity of pterygospermin.

Time after mixing, in hours.	Dilution of mixture I in broth.					Dilution of mixture II in broth.				Dilution of mixture III in broth.			Control.	
	1 75,000	1 100,000	1 225,000	1 300,000	1 300,000	1 75,000	1 150,000	1 200,000	1 300,000	1 80,000	1 200,000	1 300,000	1 200,000	1 300,000
	—	—	—	++	++	—	—	—	++	—	—	++	—	++
	—	—	—	++	++	—	—	—	++	—	—	++	—	++
0	—	—	—	++	++	—	—	—	++	—	—	++	—	++
2	—	—	—	++	++	—	—	—	++	—	—	++	—	++
4	—	—	—	++	++	—	—	—	++	—	—	++	—	++
8	—	—	—	++	++	—	—	—	++	—	—	++	—	++
24	—	—	—	++	++	—	—	—	++	—	—	++	—	++
48	—	—	—	++	++	—	—	—	++	—	—	++	—	++

— indicates no growth ; ++ indicates growth as much as in control.

Mixture I in Table V contains 0.5 mg. of cysteine per mg. of *ptyergospermin*, mixture II contains 5 mg., and mixture III 10 mg. for every mg. of *ptyergospermin*.

TABLE VI.

Effect of thiosulphate on the antibacterial activity of ptyergospermin.

Ratio of thio- sulphate to <i>ptyergospermin</i> .	Units of <i>ptyergospermin</i> per c.c. of the mixture after incubation for :—					
	Hours.					
	0	2	4	6	24	48
1 : 2	47	47	47	46	47	47
5 : 1	47	47	48	46	46	47
10 : 1	47	46	47	47	48	47

Effect of nucleic acid on the antibacterial activity of ptyergospermin.—Nucleic acid and related compounds have been found to antagonize the antibacterial activity of different antibiotics (Pandalai and George, 1947). Based mainly on these observations a theory has been suggested to explain the mode of action of antibiotics (Pandalai and George, 1947a). Since thiol compounds were found to have no effect on *ptyergospermin* activity, experiments were carried out to find out whether nucleic acids antagonize its action. It was observed that in presence of nucleic acids the antibacterial activity of *ptyergospermin* increased considerably. The results with two pathogenic micro-organisms are brought out in Table VII :—

TABLE VII.

Effect of nucleic acid on the activity of ptyergospermin.

Test organism.	Dilution and activity of <i>ptyergospermin</i> alone.	Dilution and activity of nucleic acid alone.	Activity of nucleic acid- <i>ptyergospermin</i> mixtures.		Control.
			Nucleic acid.	<i>Ptyergospermin</i> .	
<i>S. aureus</i>	1 100,000 —	1 1,000 ++	1 1,000	1 100,000 —	
				1 200,000 —	
				1 300,000 —	

— indicates no growth ; ++ indicates growth as much as in control.

TABLE VII—concl'd.

Test organism.	Dilution and activity of <i>pterygospermin</i> alone.	Dilution and activity of nucleic acid alone.	Activity of nucleic acid- <i>pterygospermin</i> mixtures.		Control.
			Nucleic acid.	<i>Pterygospermin</i> .	
<i>S. aureus</i> ...	$\frac{1}{200,000}$ —	$\frac{1}{2,000}$ ++	++
	$\frac{1}{2,000}$	$\frac{1}{100,000}$ —	...
		$\frac{1}{200,000}$ —	...
		$\frac{1}{300,000}$ ±	...
	$\frac{1}{300,000}$ ++
<i>B. dysenteriae</i> ...	$\frac{1}{200,000}$ —	$\frac{1}{1,000}$ ++	$\frac{1}{1,000}$	$\frac{1}{200,000}$ —	...
	$\frac{1}{300,000}$ —	...
	$\frac{1}{400,000}$ —	...
	$\frac{1}{300,000}$ ++	++
	$\frac{1}{200,000}$ —	...
	$\frac{1}{400,000}$ ++	$\frac{1}{2,000}$ ++	$\frac{1}{2,000}$	$\frac{1}{300,000}$ —	...
	$\frac{1}{400,000}$ —	...

— indicates no growth; ++ indicates growth as much as in control.

DISCUSSION.

The marked antibacterial activity of *pterygospermin* against a wide range of pathogens would suggest the possibility of its therapeutic applicability in human infections. However, such a possibility can be entertained only if investigations on its toxicity now in progress prove *pterygospermin* to be sufficiently non-toxic. The experiments so far carried out give no insight regarding the mode of action

of this antibiotic. The observation that in presence of nucleic acid its activity is enhanced may perhaps increase its therapeutic importance, since it is well known that at the site of bacterial infection nucleic-acid concentrations are fairly high.

SUMMARY.

The antibacterial principle present in the roots of *Moringa pterygosperma* has been isolated. Its antibacterial activity against a few Gram-positive, Gram-negative and acid-fast bacteria has been studied. *Pterygospermin* inhibits also the growth of many fungi. Cysteine and thiosulphate, the two sulphhydryl reagents, do not affect the activity of *pterygospermin*. In the presence of small amounts of nucleic acids the activity of *pterygospermin* is considerably increased. Studies relating to toxicity and blood-level concentrations are in progress.

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INVESTIGATIONS ON PLANT ANTIBIOTICS.

Part IV.

FURTHER SEARCH FOR ANTIBIOTIC SUBSTANCES IN INDIAN MEDICINAL PLANTS.

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IN Part II of this series we have recorded (George *et al.*, 1947) the results of a search for antibacterial substances present in about a hundred of the more important Indian medicinal plants and have shown that several plants give aqueous or alcoholic extracts which possess marked antibacterial properties against test organisms such as *Staph. aureus* and *Esch. coli*. This paper contains a record of examination of another ninety plants more or less on the same plan. Various aspects regarding the extraction and testing of antibacterial substances from plants, such as the capacity of various organic solvents and other media to extract the principle, examination of plants for their antibiotic content in relation to seasonal variation, different parts of the plants, and aspects such as the diffusibility factor in relation to the magnitude of the zone of inhibition observed on the agar plates, etc., have been given particular attention in the work presented in this paper.

There is evidence of increasing interest in the search for higher plants which would be sources of active antibacterial substances as is seen from the large number of references to work on plant antibiotics in recent months. It is thus obvious that due attention has to be given to the exploitation of indigenous medicinal plants for potential antibiotic substances, the presence of which in several of them have already been reported.

In general, the choice of the plants, the method of extraction, the procedure for assay, etc., were all conducted on the same lines as described in our previous paper (George *et al.*, *loc. cit.*) unless otherwise stated.

THE EXTRACTION METHODS AND THE USE OF ORGANIC SOLVANTS.

Previous workers have used the plants as such and pressed out the juice by means of presses (Sanders *et al.*, 1945) and other methods used involve crushing with sand in a mortar and draining out the juice through fine muslin (Osborn, 1943). Saline extracts were used by Carlson *et al.* (1946). Certain workers have used steam distillates of plants (Seegal and Hoden, 1945) but in this case there is the risk of the antibiotic substance losing its activity under the conditions of extraction. Practically none seems to have used organic solvents like alcohol, acetone or chloroform which are widely used in isolation and studies of natural products, particularly plant products. Sanders *et al.* (*loc. cit.*) and others suggest that the results obtained with aqueous extracts may be taken only as a preliminary indicative one and that the use of organic solvents might give altogether different indications due to the probability of the natural product getting solubilized by the solvents. Due to these facts the usual organic solvents were used for the extraction of the principle and tests were made to see whether these extracts gave the correct indication of the antibiotic values by the cup-assay method. Ethyl alcohol, absolute alcohol 90 per cent. 80 per cent methyl alcohol, chloroform, acetone, ether petroleum, ether sulphuric, etc., were used for this purpose. Other media, such as water, saline and phosphate buffer pH 7.0, were also used to evaluate their solvent power on these antibiotic principles. In order to see whether the inhibitory zones formed in the assay plates were really due to the antibiotic and not the solvent, the solvents themselves were used side by side for the assays. The test organisms were *Staph. aureus* and *Esch. coli* and in certain special cases *B. subtilis*, *B. typhosus*, *B. dysenteriae* (Shiga), etc. In Table I are presented the results of these experiments:—

TABLE I.

Showing the relative capacity of different solvents in the extraction of the antibiotic principle from different plants.

Plant.	ZONE OF INHIBITION IN MM. BY DIFFERENT SOLVENT EXTRACTS.							
	Alcohol.	Chloroform.	Acetone.	Ether.	Petroleum ether.	Water.	Phosphate buffer.	Saline.
1. <i>Moringa pterygosperma</i>	40	90	45	90	...	12	12	12
2. <i>Datura stramonium</i> ...	22	40	Insigni- ficant.	15	14	13
3. <i>Justicia gendarussa</i> ...	21	35	16	12	13	12
4. <i>Cinnamomum zeylanicum</i>	22	30	38	34	...	14	12	12
5. <i>Carica papaya</i> ...	25	25	45	15	...	11	Insigni- ficant.	Insigni- ficant.
6. <i>Plumbago zeylanica</i> ...	26	90	34	24	...	13	11	13

It may be seen from Table I that organic solvents are superior to water or any aqueous medium like saline or buffer for the extraction of the antibiotic principle. Petroleum ether seems to be of no use, while alcohol, chloroform and

acetone and in some cases ether were found to be usually good. Alcohol appears to be the most suitable from our experience and can be chosen for routine work and in special cases chloroform and acetone. For *Moringa* root, for example, alcohol, chloroform, acetone and ether were all quite good; for *Dhatura* leaf, chloroform alone gave satisfactory extraction, while the antibiotic from *Papaya* seeds acetone extraction appeared to be the best. It is thus obvious that for large-scale extraction the choice of the solvent has to be decided by preliminary extraction trials with different solvents. Alcohol, however, possesses the unique property of extracting bulk of the natural principles and has the advantage of the extract being relatively easy to purify. Under the conditions of the test it is seen that the solvents themselves have no effect on the development of inhibitory zones on the agar plates as is seen from the control plates with solvents alone. It may be pointed out that Heatley (1944) has reported the same findings.

THE SEASONAL VARIATIONS OF THE ANTIBIOTIC CONTENTS OF PLANTS.

It is quite possible that metabolic activities vary in a more or less wide range during the different seasons of the year in the case of plants, and antibiotics being substances formed in plants as by-products of metabolic activities, the seasons themselves may have an effect on the formation and accumulation of the antibiotic in the plant. It is also possible that in a particular season there may be a tendency for the antibiotic once formed and accumulated to get decomposed or to acquire less or enhanced antibiotic properties. Such changes in nature, properties, constitution, etc., of plant products are known and the point would naturally be interesting from the standpoint of antibiotic contents in plants in relation to seasonal changes. Table II gives the results obtained on tests carried out in the case of four plants and indicates that seasonal variations in the antibiotic contents do exist:—

TABLE II.

Showing the seasonal variations in the antibacterial contents of plants.

Plant.	Time of the year when tested.	Zone of inhibition in mm.
1. <i>Moringa pterygosperma</i> (root) ...	January ...	40
	April ...	32
	August ...	35
	October ...	40
2. <i>Adenanthera pavonina</i> (leaf) ...	January ...	30
	March ...	27
	July ...	19
3. <i>Plumbago zeylanica</i> (root) ...	January ...	26
	March ...	26
	June ...	22
4. <i>Carica papaya</i> (seeds) ...	January ...	24
	April ...	28
	July ...	20

THE OCCURRENCE OF ANTIBIOTIC IN THE PLANT IN RELATION TO
DIFFERENT PARTS OF THE PLANT.

Different parts of the plants have been used in various Ayurvedic prescriptions, the roots, the root bark, the stem, the stem bark, shoot, leaves, flower, fruit, seed, etc. In some cases the whole plant is used. We have seen that the entire plant *Moringa pterygosperma* is one in which all the parts yield antibiotic principles. There is, however, considerable variation in the distribution of the antibiotic principle in the different parts of the plants and this aspect is brought out in Table III:—

TABLE III.

Variation in the distribution of the antibiotic principle in the different parts of the same plants.

Plant.	Parts examined.	Mean halo diameter in mm.	REMARKS.
1. <i>Carica papaya</i> ...	<div style="display: inline-block; vertical-align: middle;"> <div style="font-size: 2em; vertical-align: middle;">{</div> <div style="display: inline-block; vertical-align: middle;"> Leaf ... Latex ... Seeds ... Fruit ... Root ... </div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="font-size: 2em; vertical-align: middle;">{</div> <div style="display: inline-block; vertical-align: middle;"> 24 11 11 </div> </div>	In almost all cases the distribution of active principle is uneven.
2. <i>Datura stramonium</i> ...	<div style="display: inline-block; vertical-align: middle;"> <div style="font-size: 2em; vertical-align: middle;">{</div> <div style="display: inline-block; vertical-align: middle;"> Leaf ... Flower ... Fruit ... Stem ... Root ... </div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="font-size: 2em; vertical-align: middle;">{</div> <div style="display: inline-block; vertical-align: middle;"> 24 13 12 11 11 </div> </div>	
3. <i>Clerodendron inerme</i> ...	<div style="display: inline-block; vertical-align: middle;"> <div style="font-size: 2em; vertical-align: middle;">{</div> <div style="display: inline-block; vertical-align: middle;"> Leaf ... Flower ... Fruit ... </div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="font-size: 2em; vertical-align: middle;">{</div> <div style="display: inline-block; vertical-align: middle;"> 11 13 ... </div> </div>	
4. <i>Moringa pterygosperma</i> ...	<div style="display: inline-block; vertical-align: middle;"> <div style="font-size: 2em; vertical-align: middle;">{</div> <div style="display: inline-block; vertical-align: middle;"> Leaf ... Flower ... Fruit ... Root ... </div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="font-size: 2em; vertical-align: middle;">{</div> <div style="display: inline-block; vertical-align: middle;"> 21 20 23 40 </div> </div>	

EXAMINATION OF SELECTED PLANTS.

The extractions of the first 100 plants we have already reported were conducted first by alcohol and the residue by water so that only whatever principle insoluble in alcohol was extracted by water. In this case, however, fresh lots were used both for alcohol and for water so that all water-soluble principles were brought down in the aqueous extract and all alcohol-soluble principles in the alcohol extracts. As stated before, the methods of extraction, the choice of plants, treatment of the plant material, assay, etc., were all carried out as described in the previous case. The results are given in Table IV. It may be seen from these results that quite a number of plants yield both alcoholic and aqueous extracts possessing antibacterial action against the test organisms used.

TABLE IV.
Results of examination of selected plants.

Plant.	Family.	Parts examined.	MEAN ZONE DIAMETER IN MM.			
			Staph. aureus.		Esch. coli.	
			Alcohol.	Aqueous.	Alcohol.	Aqueous.
<i>Acacia sundra</i> (Karingali) ...	Leguminosæ	Root	13	12	13	12.5
<i>Aconitum heterophyllum</i> (Atridhayam).		Root	13	13	10.5	11
<i>Adenanthera pavonina</i> (Anigundumani).	Leguminosæ	Leaf	35	20	34	19
		Seeds	Insignificant	18 (diffused).	11	14 (diffused).
<i>Allamanda grandiflora</i> ...	Apocynaceæ	Bark	13	...	13	12
<i>Allangium lamarekii</i> (Alangi) ...	Cornaceæ	Leaf	14	...	13	...
		...	13 (diffused).	...	23	12
<i>Alor littoralis</i> ...	Liliaceæ	Fleshy leaf	...	13 (not clear).	...	11
<i>Alpinia calcarata</i> (Vellaratha) ...	Scitamineæ	Stem	13	13	10.5	12
<i>Alpinia gulara</i> (Chittaratha) ...	"	Stem	40	Insignificant	40 (diffused).	Insignificant.
<i>Amaranthus spinosus</i> (Cherucherra).	Compositæ	Whole plant	...	13 (not clear).	...	15 (not clear).
<i>Amorphophallus campanulatus</i> (Kattu chenn).	Aroidæ	Stem	12
<i>Andropogon paniculatus</i> ...	Acanthaceæ	Leaf	16	15 (not clear).	10	12

TABLE IV—*contd.*

Plant.	Family.	Parts examined.	MEAN ZONE DIAMETER IN MM.			
			<i>Staph. aureus.</i>		<i>Esch. coli.</i>	
			Alcohol.	Aqueous.	Alcohol.	Aqueous.
<i>Argyrea speciosa</i> ...	<i>Convolvulaceæ</i>	Leaf	12
<i>Aristolochia indica</i> (Iswari)	<i>Aristolochiaceæ</i>	Root	33	...	22	15
<i>Artemesia indica</i> ...	<i>Compositæ</i>	Whole plant	11	17	11	13
<i>Asclepias currassavica</i> ...	<i>Asclepiadaceæ</i>	Leaf	...	Insignificant
		Fruit	11
<i>Atylosia barbata</i> (Kadu-uzhunnu)	<i>Leguminosæ</i>	Whole plant	12	12
<i>Barteria cristata</i> (Karimkurinji)	<i>Acanthaceæ</i>	Root	12 (not clear).	...
<i>Bassia longifolia</i> (Kat-illupi)	<i>Sapotaceæ</i>	...	12	11	15	12
<i>Biophytum sensitivum</i> (Jhala pushpa).	<i>Geraniaceæ</i>	Leaf	11	11.5 (not clear).	Insignificant	12 (not clear).
		Flower	16
<i>Bixa orellana</i> ...	<i>Bixineæ</i>	Leaf	12	...	16	...
		Fruit	12	...	18	...
<i>Blepharistemma corymbosa</i> ...	<i>Acanthaceæ</i>	Leaf	14	11	11	13
<i>Cassia absus</i> (Kattu-muthira)	<i>Leguminosæ</i>	Root	20	11.5	11	Insignificant.
<i>Cassia sophora</i> (Ponnariveeran)	<i>Leguminosæ</i>	Root	11	14 (not clear).	11	15 (not clear).

<i>Cephaandra indica</i> (Kovai)	...	Cucurbitaceae	{ Leaf Root	12 11
<i>Cissampelos pareira</i> (Pada kilangu)	...	Menispermaceae	Root	15	...	15	11
<i>Chenopodium ambrosioides</i> (Mexican tea)	...	Chenopodiaceae	Leaf	...	12 (not clear).	...	14 (not clear).
<i>Citrullus colocynthis</i> (Tumatti)	...	Cucurbitaceae	Root	11	...	13	11
<i>Clonme felina</i>	Capparidaceae	Whole plant	15	...	12	...
<i>Commelia obliqua</i> (Sahasramooli)	...	Commelinaceae	Root	18 (not clear).	12	12	12
<i>Coryliis govaniiana</i> (Eruveli)	...	Fumariaceae	{ Root and shoot.	{ 12 (very clear). 15 (not very clear).	11	12	12 (not very clear).
<i>Curculigo orchitodes</i>	Amaryllidaceae	Root tuber	14	...	10.5	...
<i>Desmodium gangeticum</i> (Gitanam).	...	Leguminosae	Root	11	13
<i>Elephantopus scaber</i> (Anashovadi)	...	Compositae	Whole shoot	11	11	15	...
<i>Emblia ribes</i> (Vayu vilangam)	...	Myrsinaceae	Seed	12.5	12.5	12	12
<i>Eragrostis cynosuroides</i>	Gramineae	Whole shoot	...	12 (not clear).	...	15
<i>Erythrina indica</i>	Leguminosae	{ Leaf Bark	{ 15.5 10.5	{ 10 11.5	{ 13.5 10.5	{ 13 13
<i>Ficus religiosa</i> (Asvattha)	...	Urticaceae	Bark	Insignificant	12	11	13
<i>Gloriosa superba</i> (Menthoni)	...	Liliaceae	Root	12	10.5	Insignificant	Insignificant
<i>Glycosmis pentaphylla</i> (Panchi)	...	Rutaceae	...	15	12	13 (not clear).	12

TABLE IV—*contd.*

Plant.	Family.	Parts examined.	MEAN ZONE DIAMETER IN MM.			
			<i>Staph. aureus.</i>		<i>Esch. coli.</i>	
			Alcohol.	Aqueous.	Alcohol.	Aqueous.
<i>Gossypium herbaceum</i> (Paruthi) ...	<i>Malvaceæ</i>	Leaf	18	...	12	12
		Flower	19	...	14	(diffused).
		Bark	17	...	15	...
<i>Hibiscus abelmoschus</i> (Kamakasturi).	"				(diffused).	13
<i>Hibiscus esculentus</i> (Venda kay) ...	"	Leaf (tender)	15	13	12	11
<i>Hydnocarpus wightiana</i> (Mara-vetti)	<i>Bixineæ</i>	Fruit	45
		Fruit rind	25
<i>Ichnocarpus frutescens</i> (Palvalli) ...	<i>Apocynaceæ</i>	Seed	19	...	10	...
<i>Ipomœa turpethum</i> (Shivadai) ...	<i>Convolvulaceæ</i>	Root	35	...	18	...
<i>Ipomœa digitata</i> (Palmuthak) ...	<i>Convolvulaceæ</i>	...	10	10	11.5	12
<i>Jasminum rottlerianum</i> (Kattumulla).	<i>Oleaceæ</i>	Root	13	12
		Root	25	Insignificant
<i>Jatropha glandulifera</i> (Avanak) ...	<i>Euphorbiaceæ</i>	Leaf	11	Enhanced growth.
		Fruit	14.5
		Root	14.5	12 (diffused).	11	12 (diffused).
<i>Jeniosporum prostratum</i> (Gnazhal poovu).	<i>Labiataæ</i>	Flower	16	17	14	15.5

<i>Kempferia galanga</i> (Kachola kilangu).	Scitamineæ	Root	12	12
<i>Kempferia rotunda</i> (Chenchu neer)	"	Root	13	13
<i>Mangifera indica</i> (Mam-maram) ...	Anacardiaceæ	Leaf	11	Diffused	11	Insignificant.
		Leaf	12	...
		Bark
		Fruit	17	Insignificant	Diffused	...
<i>Mitchelia champaca</i> (Chempuk) ...	Magnoliaceæ	Root	11	...
		Leaf	12	12	13	...
		Leaf	Insignificant	12	Insignificant	12
		Bark	10.5	13 (not clear).	10	16
<i>Mimusops elengi</i> ...	Sapotaceæ	Root	11	...	Insignificant	...
		Root	17	...	15	...
		Bark	13
		Root	14	13	13	16
		Stem	11	...	10.5	...
		Leaf	...	12	...	12.5
		Fruit	...	11
		Flower	13	13	11	13
		Root	20	11	12	12
<i>Oroxylon indicum</i> (Vangamarum)	Bignoniaceæ	Root	12	13
<i>Pavonia sylhanica</i> ...	Malvaceæ	Root	12	15	11	11

TABLE IV—concl'd.

Plant.	Family.	Parts examined.	MEAN ZONE DIAMETER IN MM.			
			<i>Staph. aureus.</i>		<i>Esch. coli.</i>	
			Alcohol.	Aqueous.	Alcohol.	Aqueous.
<i>Piscolus adenanthus</i> (Katu-payaru).	<i>Leguminosæ</i>	Whole plant	12	13 (not clear).	13	17
<i>Phyllanthus emblica</i> ...	<i>Euphorbiaceæ</i>	Fruit	26	...	14	Insignificant.
<i>Phyllanthus urinaria</i> ...	"	Weak stem (vulli).	Insignificant.
<i>Picrasma quassioides</i> (Cheruthoku)	<i>Simarubææ</i>	Root	20	Insignificant	28	11
<i>Premna interfolia</i> (Munnay)	<i>Verbenacææ</i>	Root	15	13.5	12.5	13
<i>Pseudarthwa viscidia</i> (Moovila)	"	Root	12.5	12	14	13.5
<i>Psoralea corylifolia</i> (Bogi vittulu)	"	Seed	13.5 (not clear).	14	13 (not clear).	12.5
<i>Pterocarpus santalinus</i> (Rakta-chandanum).	"	Stem	14	Insignificant	12	11
<i>Pterocarpus marsupium</i> (Vengai)	"	Inner stem	45	12	25	14
<i>Raphanus sativus</i> (Mullanki)	<i>Crucifereæ</i>	Root	13	Insignificant.
<i>Rumusatia vivipara</i> (Maravara chempu).	<i>Aroidææ</i>	Root	45	20	13	...
<i>Sansevieria cylindrica</i> ...	<i>Hamnodoracææ</i>	Fleshy leaf	11	...
<i>Sansevieria zeylanica</i> ...	"	"	11	12 (not clear).
<i>Saraca indica</i> ...	<i>Leguminosææ</i>	Leaf	12	...	12	12

Plant	Local Name	Family	Part	45	12	Insufficient	Insufficient.
<i>Sasura lappa</i> (Kottam)	...	Compositæ	Root	45	12		Insufficient.
<i>Semicarpus anacardium</i> (Shenkottai).	...	Anacardiaceæ	Seed	...	12	15 (not clear).	...
<i>Solanum melongena</i> (Kathiri-kai)	...	Solanaceæ	Root	11	11	...	12
<i>Solanum furvum</i>	"	Leaf	Insufficient	Insufficient	12	12 (not clear).
<i>Solanum verbasifolium</i>	"	Leaf	"	14 (not clear).
<i>Stereospermum suarcolens</i> (Pathiri)	...	Bignoniaceæ	Root	19	...	11	12
<i>Streblus asper</i> (Methli)	...	Urticaceæ	Leaf	13	Insufficient	12	Insufficient.
			Bark	12	12	11	13
			Root	11
			Leaf	11	11	...	13
<i>Tamarindus indicus</i> (Puli)	...	Leguminosæ	Fruit	...	14 (diffused).	11	13
			Root bark
<i>Terminalia arjuna</i> (Vella-marda)	...	Combretaceæ	Insufficient	...	Insufficient.
<i>Thespesia populanea</i> (Poovarasi)	...	Malvaceæ	Leaf	12	12	14	"
<i>Trichosanthes cucumerina</i> (Kadupadavalla).	...	Cucurbitaceæ	Whole plant	12	11	13 (diffused).	13
<i>Ventago madras patana</i> (Vempadon).	...	Rhamnaceæ	Bark	13	12	10	12
<i>Vitex pectuncularia</i> (Navaladi)	...	Verbenaceæ	Leaf	18	...	13	12 (not clear).
<i>Wrightia tinctoria</i>	Apocynaceæ	Leaf	Insufficient	12

DISCUSSION.

Higher plants do seem to offer great chance for economic production of substances possessing strong antibacterial properties. Certain plants give alcoholic extracts which completely inhibit the growth of the test organisms giving a sterile plate (90 mm.). In general, the plant antibiotic substances appear to be more inhibitory to Gram-positive organisms than to the Gram-negative type. It may be remembered that penicillin and some of the other prominent antibiotic agents of fungal origin are also rather selective in their inhibitory action, most of them being inhibitory to Gram-positive organisms. As reported in the previous paper it was noticed that some reputed medicinal plants used extensively in the cure of bacterial diseases possess no pronounced antibiotic activity quite contrary to expectation and their therapeutic action has to be traced to other causes. Probably, compounds may be present in the extract which have properties antagonistic to the inhibitory substances, cysteine for example, and which, therefore, might mask or counteract the antibacterial properties. Better processing, however, may avoid such inhibitory substances from being present and exerting their action. Tests on wider range of infectious organisms have to be undertaken instead of confining to one or two test organisms. Extracts which are without action on *Staph. aureus*, for example, may be inhibitory to *B. typhosus* or *B. dysenteriae*. Indeed it was found that the extract of the plant *Hediotis* was inhibitory to *Vibrio cholerae*, while it was insensitive to *Staph. aureus* or *Esch. coli* and the brinjal plant gave an extract inhibitory to *B. typhosus* but not to *Esch. coli* or *Staph. aureus* and so on.

SUMMARY.

1. The preliminary survey of antibacterial substances present in well-known Indian medicinal plants reported in the previous paper has been continued to include 90 more plants. The results show that many plants give alcoholic or aqueous extracts possessing strong antibacterial properties, some on Gram-positive organisms, and some on Gram-negative organisms.

2. Among organic solvents alcohol was found to be the best solvent extracting most of the antibiotic principles and also possesses the added advantage of the extract being relatively easy to purify. Chloroform and acetone also afford good extraction in certain instances.

3. Though the diffusibility of the test liquid is a factor among others in the formation of inhibitory zones on the agar plates, in general, reliable values of the intrinsic antibacterial activity of a test liquid is obtained by the agar plate cup assay.

4. A slight seasonal variation in the antibiotic contents in various plants has been observed.

5. The distribution of the antibiotic principle in the various parts of the plants is quite uneven. While in some cases like *Moringa pterygosperma* the entire plant is a good source of antibiotic principles, in several, the principle was found abundant either in the roots, stem, leaves, fruits or flowers and often in the seeds.

6. In general, it may be said that plant antibiotics are more inhibitory to Gram-positive pathogens than to Gram-negative pathogens. The latter are also

inhibited by quite a good number of extracts and a few are inhibitory to both types.

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CAROTENOID PIGMENTS OF DIFFERENT CROSS-BREEDS OF
CULTIVATED AND WILD VARIETIES OF TOMATOES
AND THE EFFECT OF FURTHER INTER-
VARIETAL CROSSING ON THE
CAROTENOID CONTENT.

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EARLIER studies on tomatoes in relation to vitamin A are reported by Osborne and Mendel (1920), Mendel (1920), Sherman and Munsell (1925) and Davis and Stillmann (1926). Observations on the carotenoids of tomato have also been made by Shivrina (1937, 1938), Ott (1938), Went, LeRosen and Zechmeister (1942), Hamner and Ellis (1943), LeRosen, Went and Zechmeister (1941) and Zechmeister, LeRosen, Went and Pauling (1941). These authors have noticed considerable differences in carotenoid content in different samples of the fruit and even from different fruits picked from the same vine at comparable stages of ripeness. Zechmeister *et al.* (*loc. cit.*) found in the tomato, besides lycopene and β -carotene, several other pigments, namely, neo-lycopene A, neo-lycopene B, γ -carotene, α -carotene, prolycopene and still some unidentified carotenoids. A carotenoid named ζ -carotene has been isolated by Nash and Zscheile (1946) and Zscheile and Porter (1947). These authors examined numerous breeds and crosses of tomato and isolated in addition to β -carotene and lycopene, neo- β -carotene B,

neo- β -carotene U, γ -carotene, α -carotene, -carotene and isomers of lycopene, namely, neo-lycopene A and neo-lycopene B. One of the striking observations they made was that in many species of tomatoes whose fruit is yellow or green in colour, lycopene is present only in traces, while it goes up to 300 to 400 $\mu\text{g./g.}$ in some of the dark-red varieties. LeRosen, Went and Zechmeister (*loc. cit.*) also studied carotenoid pigments in relation to genes.

In this investigation, the carotenoid pigments of some cross-breeds of the cultivated and wild varieties of tomatoes have been investigated together with the effect of further inter-varietal crossing on the carotenoid content. The numerous selfs and crosses were produced from the true species—*Lycopersicon esculentum* and *Lycopersicon pimpinellifolium*. It was anticipated that this investigation will lead to results of practical importance from the point of view of obtaining cultivated varieties containing high amounts of β -carotene and thereby more of vitamin A. The study is also of value from the fundamental aspects of carotenoid physiology in relation to pigment development in plants.

The samples examined were obtained through the courtesy of the Economic Botanist, Indian Institute of Agricultural Research, Delhi, where these varieties and their cross-breeds were grown. All the varieties and their cross-breeds were grown under identical conditions of manuring, irrigation and climatic factors. Samples of average-sized fruit, selected at random, were examined at different stages of ripeness. Four stages of ripeness were recognized:—

- (i) At one-quarter ripe stage when the fruit was all green in colour.
- (ii) At one-half ripe stage when the fruit had lost most of its green colour.
- (iii) At three-quarters ripe stage when the fruit was not quite uniformly coloured.
- (iv) At fully ripe stage when the fruit was completely coloured.

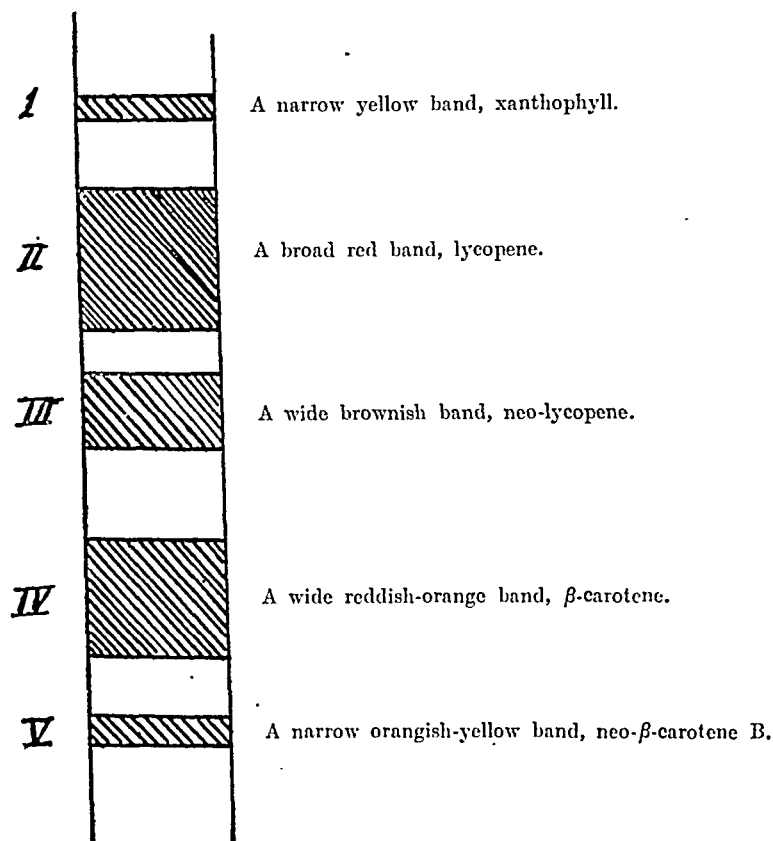
Fresh unpeeled fruits were used for analysis. Generally the whole fruit was taken but in case of large fruits, alternate equatorial slices were used.

METHOD OF ANALYSIS.

The procedure followed for the complete extraction, isolation and estimation of the carotenoid pigments is described in the next paper (Sadana and Ahmad, 1949, p. 193 *this issue*). The petroleum ether extract containing all the carotenoid pigments were directly introduced on an activated alumina column. The pigments after separation were estimated colorimetrically by means of a standard curve of β -carotene against 0.04 per cent potassium dichromate. The same curve was used for the estimation of all the pigments except for lycopene and neo-lycopene. The amounts of lycopene and neo-lycopene were determined by matching the solutions against 0.001 per cent methyl orange, the quantities being read off from a standard reference curve showing the relation between the colour intensities of different concentrations of pure lycopene and 0.001 per cent methyl orange. The results are expressed as $\mu\text{g./g.}$ The vitamin A potency is expressed in I.U. per g.

The chromatograms of all tomato varieties showed the presence of five distinct bands. A typical Chromatogram is given here :—

A TYPICAL CHROMATOGRAM OF THE PIGMENTS OF TOMATO FRUIT.



The characterization of the various bands constituting the different pigments is given below :—

1. The pigment of this band was taken to be xanthophyll as was shown by mixed chromatography and as it could be removed by phasic separation with methanol.

2. This was identified as lycopene by its absorption spectra.

3. This represents neo-lycopene, an isomer of lycopene.

4. The pigment of this band was identified as β -carotene by its absorption spectra.

5. This represents an isomer of β -carotene, called neo-carotene B by Polgar and Zechmeister (1942).

The analytical data for the different carotenoid pigments are presented in Tables I and II. The former gives the carotenoid content of the parent varieties

TABLE I.
Carotenoid content of parent species of tomato fruits.

Species.	Stage of ripeness.	Sum of individual pigments. μg./g.	Xantho- phyll. μg./g.	Lycopene. μg./g.	Neo- lycopene. μg./g.	β- carotene. μg./g.	Neo-β- carotene B. μg./g.	Total active pigments in terms of β- carotene. μg./g.	Vitamin A potency. I.U. per g.
T ₈₁	1	13.9	4.4	4.8	...	3.9	0.8	4.3	7.2
	1	19.5	5.8	10.2	...	2.6	0.9	3.05	5.1
	2	40.0	5.3	21.1	5.0	7.4	1.2	8.0	13.3
	3	89.1	5.1	51.8	16.9	13.1	2.2	14.2	23.7
	4	134.6	4.5	80.7	31.1	7.6	1.7	8.45	14.1
T ₈₁₇	1	13.9	3.9	6.1	...	2.7	1.2	3.3	5.5
	2	27.6	3.5	16.8	2.0	3.9	1.4	4.6	7.7
	2	29.9	4.0	16.0	4.9	3.8	1.2	4.4	7.3
	3	106.6	4.7	60.8	23.3	6.8	2.0	7.8	13.0
	4	253.5	4.2	181.8	54.8	10.5	2.2	11.6	19.3
T ₈₇	1	8.3	4.3	1.4	...	2.6	...	2.6	4.3
	2	18.3	3.6	8.3	...	4.4	2.0	5.4	9.0
	2	18.8	2.9	7.2	0.6	5.9	2.2	7.0	11.7
	3	33.6	2.7	20.9	2.6	5.9	1.5	6.65	11.1
	4	108.6	3.2	74.0	23.2	6.7	1.5	7.45	12.4

T ₂	1	8.7	4.1	2.1	...	2.5	...	2.5	4.2
	2	33.7	5.1	17.2	6.0	4.8	0.7	5.15	8.6
	2	33.8	4.3	16.0	7.7	5.1	0.7	5.45	9.1
	3	54.5	3.5	31.5	11.1	7.5	0.9	7.95	13.3
	4	103.0	7.0	63.6	14.6	16.6	1.2	17.2	28.7
Earliana	1	5.5	1.9	1.5	...	1.7	0.4	1.9	3.2
	1	12.2	2.5	4.1	2.0	3.0	0.6	3.3	5.5
	2	41.9	2.9	21.8	8.4	6.9	1.9	7.85	13.1
	3	43.5	3.8	25.6	6.3	5.7	2.1	6.75	11.3
	4	90.9	3.4	62.1	17.3	6.6	1.5	7.35	12.3

TABLE II.
Carotenoid content of crossed varieties of tomatoes.

Species.	Stage of ripeness.	Sum of individual pigments. $\mu\text{g./g.}$	Xantho- phyll. $\mu\text{g./g.}$	Lycopene. $\mu\text{g./g.}$	Neo- lycopene. $\mu\text{g./g.}$	β - carotene. $\mu\text{g./g.}$	Neo- β - carotene B. $\mu\text{g./g.}$	Total active pigments in terms of β - carotene. $\mu\text{g./g.}$	Vitamin A potency. I.U. per g.
$\text{Ts}_1 \times \text{Ts}_{17}$	1	12.9	5.1	4.5	...	3.0	0.3	3.15	5.3
	1	15.4	5.7	5.1	1.3	2.3	1.0	2.8	4.7
	2	48.6	3.4	28.7	10.0	4.0	2.5	5.25	8.8
	2	59.8	3.3	35.3	12.5	6.5	2.2	7.6	12.7
	3	101.9	3.6	73.2	18.1	5.7	1.3	6.35	10.6
	4	197.7	4.8	135.9	46.5	8.7	1.8	9.6	16.0
	1	7.6	3.0	2.4	...	2.2	...	2.2	3.7
	2	26.1	3.1	16.0	...	5.4	1.6	6.2	10.3
$\text{Ts}_{17} \times \text{Ts}_1$	3	41.1	3.2	26.0	5.6	4.7	1.6	5.5	9.2
	3	58.4	2.6	34.3	14.6	5.2	1.7	6.05	10.1
	4	166.6	4.6	111.1	42.0	6.9	2.0	7.9	13.2
	1	17.9	4.3	8.6	...	3.9	1.1	4.45	7.4
	2	30.8	4.0	15.0	4.7	5.1	2.0	6.1	10.2
	2	39.5	3.3	23.5	7.0	4.0	1.7	4.85	8.1
	3	103.5	2.6	65.0	25.4	8.9	1.6	9.7	16.2
	4	193.4	3.8	143.1	35.0	9.7	1.8	10.6	17.7
$\text{Ts}_1 \times \text{Ts}_7$	1	12.9	5.1	4.5	...	3.0	0.3	3.15	5.3
	1	15.4	5.7	5.1	1.3	2.3	1.0	2.8	4.7
	2	48.6	3.4	28.7	10.0	4.0	2.5	5.25	8.8
	2	59.8	3.3	35.3	12.5	6.5	2.2	7.6	12.7
	3	101.9	3.6	73.2	18.1	5.7	1.3	6.35	10.6
	4	197.7	4.8	135.9	46.5	8.7	1.8	9.6	16.0
	1	7.6	3.0	2.4	...	2.2	...	2.2	3.7
	2	26.1	3.1	16.0	...	5.4	1.6	6.2	10.3
$\text{Ts}_{17} \times \text{Ts}_7$	3	41.1	3.2	26.0	5.6	4.7	1.6	5.5	9.2
	3	58.4	2.6	34.3	14.6	5.2	1.7	6.05	10.1
	4	166.6	4.6	111.1	42.0	6.9	2.0	7.9	13.2
	1	17.9	4.3	8.6	...	3.9	1.1	4.45	7.4
	2	30.8	4.0	15.0	4.7	5.1	2.0	6.1	10.2
	2	39.5	3.3	23.5	7.0	4.0	1.7	4.85	8.1
	3	103.5	2.6	65.0	25.4	8.9	1.6	9.7	16.2
	4	193.4	3.8	143.1	35.0	9.7	1.8	10.6	17.7

$T_{31} \times T_{31}$	1	15.3	5.1	5.2	1.7	2.7	0.6	3.0	5.0
	2	34.9	3.9	18.7	6.6	5.1	0.6	5.4	9.0
	2	44.3	5.9	22.2	5.3	8.8	2.1	9.85	16.4
	3	103.3	5.5	57.3	29.9	7.8	2.8	9.2	15.3
$T_{317} \times T_{317}$	4	148.2	7.0	87.0	39.5	11.2	3.5	12.95	21.6
	1	7.1	1.3	3.9	...	1.9	...	1.9	3.2
	2	33.6	2.8	16.9	6.4	5.9	1.6	6.7	11.2
	3	68.4	2.6	41.5	9.3	8.3	1.7	9.15	15.3
$T_{317} \times T_{317}$	4	121.7	3.9	90.0	17.3	8.8	1.7	9.65	16.1
	1	5.1	1.6	2.5	...	1.0	...	1.0	1.7
	2	20.6	1.8	9.4	2.8	4.9	1.7	5.75	9.6
	2	29.2	1.7	16.4	4.0	5.7	1.4	6.4	10.7
$T_{317} \times T_{317}$	3	65.7	2.1	37.8	17.5	6.6	1.7	7.45	12.4
	4	141.8	3.4	99.8	30.6	5.6	2.4	6.8	11.3
	1	7.5	2.4	2.4	...	2.2	0.5	2.45	4.1
	2	11.4	1.8	5.8	...	3.4	0.4	3.6	6.0
$T_{317} \times E_{317}$	2	17.5	2.0	4.3	1.2	8.6	1.4	9.3	15.5
	3	60.6	3.3	37.5	14.8	9.3	1.7	10.15	16.9
	4	183.5	3.5	141.1	29.7	7.7	1.5	8.45	14.1

which represent fixed selections from an inter-specific cross between the cultivated varieties, *Lycopersicon esculentum* and the wild varieties, *Lycopersicon pimpinellifolium*. This includes Ts₁, Ts₄, Ts₇, Ts₁₇. These varieties were further crossed and the results of these inter-varietal crosses are reported in Table II.

DISCUSSION.

Pigments isolated and identified in the different cross-breeds of tomatoes include xanthophyll, lycopene, neo-lycopene, β -carotene and neo- β -carotene B only. No additional bands appeared. Consequently, α -carotene γ -carotene, and pro-lycopene reported by Zechmeister *et al.* (*loc. cit.*) and ζ -carotene reported by Nash and Zscheile (*loc. cit.*) and Zscheile and Porter (*loc. cit.*) were not discovered or identified.

The concentrations of lycopene and β -carotene varied greatly in the many species examined. Neo-lycopene concentration is principally a function of the lycopene concentration. Usually 1 μ g. of neo-lycopene is found to every 3 μ g. to 4 μ g. of all trans-lycopene. Neo- β -carotene B, the isomer of β -carotene, too is a function of β -carotene content. About 15 per cent to 25 per cent of β -carotene fraction obtained by chromatographic separation is neo- β -carotene B.

The data in Table I would indicate that the carotenoid content of the parent tomato species could be divided into three categories:—

(i) The one which contain high amounts of lycopene and its isomer, neo-lycopene, but low amounts of β -carotene and its isomer, neo- β -carotene B. As for example is the case with tomato species Ts₁₇. This species contains the highest amount of lycopene, neo-lycopene and thus the total carotenoid pigments being 6.1 to 181.8 μ g./g., 0.0 to 54.8 μ g./g. and 13.9 to 253.5 μ g./g. respectively, but β -carotene and its isomer, neo- β -carotene B, are present to the extent of 2.7 to 10.5 μ g./g. and 1.2 to 2.2 μ g./g. The vitamin A potency has been found to vary between 5.5 and 19.3 I.U. per g.

(ii) Secondly, the one with low amounts of lycopene and its isomer but with comparatively higher amounts of β -carotene and its isomer as is found in the case of species Ts₁ and Ts₄. In these two species, the amounts of lycopene, neo-lycopene and the total carotenoid pigments vary between 4.8 to 89.7 μ g./g., 0.0 to 31.1 μ g./g. and 13.9 to 134.6 μ g./g. for Ts₁ species and 2.1 to 63.6 μ g./g., 0.0 to 14.6 μ g./g. and 8.7 to 103.0 μ g./g. respectively for Ts₄ species. β -carotene and neo- β -carotene B in these two species are present to the extent of 2.6 to 13.1 μ g./g. and 0.8 to 2.2 μ g./g. for Ts₁ and 2.5 to 16.6 μ g./g. and 0.0 to 1.2 μ g./g. for Ts₄ respectively.

The vitamin A potency shows a variation range from 5.1 to 23.7 I.U. per g. in the case of Ts₁ species and 4.2 to 28.7 I.U. per g. in the case of Ts₄ species.

(iii) Thirdly, the one which contain low amounts of both lycopene and its isomer as well as β -carotene and its isomer as is the case with the species Ts₇ and *Earliana*. In species Ts₇, the amount of lycopene, neo-lycopene and the total carotenoid pigments vary between 1.4 to 74.0 μ g./g., 0.0 to 23.2 μ g./g. and 8.3 to 108.6 μ g./g. respectively, while that of β -carotene and neo- β -carotene B between 2.6 to 6.7 μ g./g. and 0.0 to 2.2 μ g./g. respectively. In species *Earliana* likewise, lycopene, neo-lycopene and the total carotenoid pigments are found to the

extent of 1.5 to 62.1 $\mu\text{g./g.}$, 0.0 to 17.3 $\mu\text{g./g.}$, and 5.5 to 90.9 $\mu\text{g./g.}$, respectively, whereas β -carotene and its isomer neo- β -carotene B are present to the extent of 1.7 to 6.9 $\mu\text{g./g.}$ and 0.4 to 2.1 $\mu\text{g./g.}$ respectively.

The vitamin A potency in these two species, Ts_1 and *Earliana*, vary between 4.3 to 12.4 I.U. per g. and 3.2 to 13.1 I.U. per g.

The amount of xanthophyll, however, could not be correlated to any of the species.

A comparative study of Tables I and II reveals some interesting results on the effect of crossing on the lycopene and β -carotene content of tomatoes. The data indicate that lycopene content of the crosses, in general, is intermediate between the two parent species but the quantity of β -carotene and thereby the vitamin A potency of the fruit is depressed and is lower than either of the two parent species. The crossed species $Ts_1 \times Ts_{17}$ and $Ts_{17} \times Ts_1$ have a lycopene content of 135.9 $\mu\text{g./g.}$ and 111.1 $\mu\text{g./g.}$ and a β -carotene content of 8.7 $\mu\text{g./g.}$ and 6.9 $\mu\text{g./g.}$, while the parent species Ts_1 and Ts_{17} have a lycopene content of 89.7 $\mu\text{g./g.}$ and 181.1 $\mu\text{g./g.}$ and a β -carotene content of 13.1 $\mu\text{g./g.}$ and 10.5 $\mu\text{g./g.}$ respectively. The vitamin A potency of the parent species Ts_1 and Ts_{17} is 23.7 and 19.3 I.U. per g. respectively, while that of crossed species $Ts_1 \times Ts_{17}$ and $Ts_{17} \times Ts_1$ is 16.0 and 13.2 I.U. per g. respectively, which is lower than either of the two parent species.

Similar is the case with the crosses $Ts_1 \times Ts_4$, $Ts_{17} \times Ts_4$ and $Ts_{17} \times Ts_7$. In these, lycopene is present to the extent of 87.0, 90.0 and 99.8 $\mu\text{g./g.}$ and β -carotene to the extent of 11.2, 8.8 and 6.6 $\mu\text{g./g.}$ respectively. The vitamin A potency in these crosses is found to be 21.6, 16.1 and 12.4 I.U. per g. respectively. The values of lycopene are intermediate between the two parent species and the vitamin A potencies are lower than either of the crossed species.

In the case of the crossed species $Ts_{17} \times \textit{Earliana}$, both lycopene and β -carotene have intermediate values as compared to the two parent species and the quantity of β -carotene is not lower than either of the species as is the case with other crosses. In this, lycopene and β -carotene are found to be 141.1 and 9.3 $\mu\text{g./g.}$ respectively, whereas the parent species Ts_{17} and *Earliana* have a lycopene content of 181.8 and 62.1 $\mu\text{g./g.}$ and a β -carotene content of 10.5 and 6.9 $\mu\text{g./g.}$ respectively. The vitamin A potencies of the parent species Ts_{17} and *Earliana* are 19.3 and 13.1 I.U. per g., while that of the crossed species is 16.9 I.U. per g.

In the case of crossed species $Ts_1 \times Ts_7$, however, it is found that the amount of lycopene is greater (and not intermediate) than either of the two parent species, whereas β -carotene is intermediate (and not lower) as compared to the two parent species. In this cross, lycopene is present to the extent of 143.1 $\mu\text{g./g.}$ and β -carotene to the extent of 9.7 $\mu\text{g./g.}$, while the parent species Ts_1 and Ts_7 have a lycopene content of 89.7 and 74.0 $\mu\text{g./g.}$ and a β -carotene content of 13.1 and 6.7 $\mu\text{g./g.}$ respectively. The vitamin A potency of the crossed species is 17.7 I.U. per g., while that of parent species is 23.7 and 12.4 I.U. per g. respectively.

SUMMARY.

The carotenoid pigments and the vitamin A activity of some cross-breeds of cultivated and wild varieties of tomatoes and the effect of further inter-varietal

crossing on the carotenoid content have been investigated. The concentrations of lycopene and β -carotene varied greatly in the many species examined. Some species contain higher amounts of lycopene with low amounts of β -carotene, while others with comparatively lower amounts of lycopene but higher amounts of β -carotene and still others with comparatively lower amounts of both lycopene and β -carotene.

In inter-variatal crossing, generally lycopene has intermediate values as compared to the parent species, whereas β -carotene content is depressed. In the case of the crosses $Ts_1 \times Ts_7$ and $Ts_{17} \times Earliana$, however, β -carotene content also increases and has intermediate values as compared to the parent species.

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METABOLISM OF THE CAROTENOID PIGMENTS OF THE MANGO DURING THE DEVELOPMENT OF THE FRUIT.

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THE carotenoid pigments of a number of different varieties of mangoes have been reported earlier (Sadana and Ahmad, 1946). It was found that the vitamin A content of mangoes varied widely from one variety to the other. Mango fruit generally is not allowed to ripen on the tree as it is liable to drop down before it is ripe and thus get spoiled. It is also open to the attack of birds. Therefore, there is a tendency on the part of the growers to pluck the fruit soon after it is mature to avoid losses from storms, insects, birds and other pests. After plucking, it ripens on storage under hay, the temperature rising to a range between 30°C. and 42°C. Under these conditions the cellular activity continues and the ripening changes take place.

Mango is one of the most valued tropical fruits and is a rich source of vitamin A. Earlier observations on the increase in the total hydrocarbon pigments of mango during ripening have been made by De (1937), Banerjee and Ramasarma (1938) and Ramasarma and Banerjee (1940). We have now investigated the changes that occur in individual carotenoid pigments during ripening and have also studied the factors that influence the vitamin A content of the fruit.

The following three varieties of mangoes were chosen for the investigations reported in this paper:—

- (i) *Saroli* mango which was found to be the variety richest in carotenoids.
- (ii) *Chounsa* mango which is moderately rich.
- (iii) *Desi* mango which is also moderately rich in carotenoids.

EXPERIMENTAL.

The procedure followed for the complete extraction and isolation of the various carotenoid pigments is described in an earlier paper (Sadana and Ahmad, *loc. cit.*).

The different pigments isolated and identified from *Saroli* mangoes were xanthophyll, β -carotene, neo- β -carotene U and neo- β -carotene B, but in the case of *Desi* variety mangoes, neo- β -carotene U was found to be absent but one additional band of neo-xanthophyll was detected. A typical chromatogram and the detailed characterization of the various carotenoid pigments constituting the different bands are also described in that paper (Sadana and Ahmad, *loc. cit.*).

Saroli MANGOES.

The first lot of *Saroli* mangoes was plucked in the first week of June, when the mangoes were still growing in size and were completely unripe. The second lot of *Saroli* mangoes was plucked near the end of the month when the fruits had reached the mature-green stage and when they are usually removed from the tree by the growers. They were still completely unripe. The first lot consisted of 20 mangoes and the second lot of 30 mangoes. All the mangoes were plucked carefully, were almost equal in size and were obtained from the same tree. These were kept in a wooden box and allowed to ripen at room temperature.

Samples were taken at random and determinations were made for the individual carotenoid content of the pulp during the 4th, 6th, 8th, 10th, 11th, 12th, 13th and 15th day after plucking. It was found by trial that representative samples could be had by taking longitudinal sections of the flesh for the estimation.

Analytical data regarding the individual carotenoid content of the first lot of *Saroli* mangoes which were not quite mature when plucked in the first week of June, during different stages of ripening, are given in Table I. Results of analyses for the second lot of *Saroli* mangoes, plucked at the mature-green stage near the end of June, are shown in Table II.

Desi MANGOES.

Twenty unripe mangoes of *Desi* variety of almost the same size were carefully plucked from the same tree. A batch of the fresh mangoes were immediately analysed for the various carotenoid pigments present. The remaining mangoes were kept at room temperature and analysed after every two or three days during successive stages of ripening. The results are summarized in Table III.

Chounsa MANGOES.

Forty *Chounsa* mangoes of almost the same size were plucked from a tree while these were still in an unripe state. One-half of these mangoes was kept in a wooden box at room temperature and the second half at 42°C. and allowed to ripen separately. Samples from the two lots were taken at random separately and the changes that occur in the different individual carotenoid pigments determined at successive intervals. In a few mangoes of each lot putrefaction had set in and these mangoes were discarded. The analytical data regarding the individual carotenoid pigments for the mangoes ripened at room temperature are given in Table IV and for those ripened at 42°C. in Table V.

TABLE I.
Carotenoid pigments of Sirohi mangoes during different stages of ripening on storage at room temperature.
First lot.

Serial number.	Number of days after plucking.	Total pigments, $\mu\text{g./g.}$	Xanthophyll, $\mu\text{g./g. per cent.}$	Neo- β -carotene U, $\mu\text{g./g. per cent.}$	β -carotene, $\mu\text{g./g. per cent.}$	Neo- β -carotene B, $\mu\text{g./g. per cent.}$	Total active pigments in terms of β -carotene, $\mu\text{g./g. per cent.}$	Vitamin A potency, I.U. per g.	Xanthophyll : carotene ratio.					
1	4	12.2	6.5	53.3	1.1	9.0	3.8	31.1	0.8	6.5	4.47	36.6	7.5	1.71
2	4	14.1	6.0	48.9	1.3	9.2	5.2	36.9	0.7	5.0	5.87	41.6	9.8	1.33
3	6	37.3	16.9	45.3	2.3	6.2	15.1	40.5	3.0	8.0	17.20	46.1	28.7	1.12
4	6	43.0	18.5	43.0	3.9	9.1	18.5	43.0	2.1	4.8	20.52	47.7	34.2	1.10
5	8	57.5	23.5	40.9	6.1	10.6	23.3	40.5	4.6	8.0	27.12	47.2	45.2	1.00
6	8	53.5	21.1	41.1	5.9	9.9	23.4	39.3	5.0	9.7	27.77	46.7	46.8	1.00
7	10	86.4	36.1	41.8	4.2	4.8	39.5	45.7	6.6	7.6	43.85	50.7	73.1	0.91
8	10	103.8	41.4	40.0	4.7	4.5	49.5	47.7	8.2	7.9	54.78	52.8	91.3	0.84
9	11	107.5	43.4	40.3	5.6	5.2	49.6	46.1	8.9	8.3	55.45	51.6	92.4	0.87
10	12	115.2	41.6	36.1	4.7	4.1	60.3	52.3	8.6	7.5	65.77	57.1	109.6	0.69
11	13	133.7	39.3	29.4	5.5	4.1	73.3	51.9	15.6	11.6	82.47	61.7	120.8	0.53
12	15	147.2	39.5	26.9	5.4	3.6	88.8	60.3	13.5	9.2	96.9	65.8	161.5	0.44

TABLE II.
Carotenoid pigments of Saroli mangoes during different stages of ripening on storage at room temperature.
Second lot.

Serial number.	Number of days after plucking.	Total pigments. $\mu\text{g./g.}$	Xanthophyll. $\mu\text{g./g. per cent.}$	Neo- β -carotene U. $\mu\text{g./g. per cent.}$	β -carotene. $\mu\text{g./g. per cent.}$	Neo- β -carotene B. $\mu\text{g./g. per cent.}$	Total active pigments in terms of β -carotene. $\mu\text{g./g. per cent.}$	Vitamin A potency. I.U. per g.	Xanthophyll: carotene ratio.					
1	4	12.5	6.7	53.6	1.1	9.0	3.9	31.2	0.8	6.3	4.57	36.6	7.6	1.72
2	4	14.5	7.0	48.3	1.4	9.6	5.3	36.5	0.8	5.5	6.05	41.7	10.1	1.32
3	4	17.1	7.4	43.2	1.1	6.4	7.2	42.1	1.4	8.2	8.17	47.8	13.6	1.01
4	5	18.8	8.0	42.5	0.8	4.2	8.0	42.6	2.0	10.6	9.20	49.0	15.3	1.00
5	5	19.8	8.1	40.6	1.2	6.0	9.3	47.3	1.2	6.0	10.20	51.5	17.0	0.87
6	5	21.6	8.6	39.7	1.2	5.7	9.4	43.7	2.4	10.8	10.90	50.5	18.2	0.91
7	6	36.3	11.0	30.3	3.3	9.1	18.9	52.0	3.1	8.5	21.28	58.6	35.5	0.58
8	6	38.6	11.3	29.3	1.3	3.3	20.8	53.9	5.2	13.5	23.72	61.4	39.5	0.54
9	6	41.8	12.2	29.1	1.2	2.9	21.3	51.0	7.1	17.0	25.15	60.2	41.9	0.57
10	6	43.4	12.6	29.0	2.1	4.8	23.9	55.1	4.8	11.0	26.82	61.8	44.7	0.53
11	6	48.6	13.3	27.4	2.5	5.1	26.2	54.0	6.6	13.5	30.12	62.0	50.3	0.51
12	8	72.7	21.2	29.2	4.6	6.2	39.1	53.8	7.8	10.7	44.15	60.7	73.6	0.51
13	9	106.7	31.6	29.6	4.7	4.1	60.3	50.5	10.1	9.5	66.52	62.3	110.8	0.52
14	9	103.9	27.9	26.9	5.4	5.2	56.5	54.3	14.1	13.6	64.90	62.4	108.2	0.49
15	9	110.1	28.3	25.7	3.7	3.3	65.1	59.1	13.0	11.8	72.52	63.8	120.9	0.43
16	10	134.1	34.6	25.8	8.1	6.0	75.7	56.5	15.7	11.7	85.57	63.8	142.6	0.46
17	10	138.7	35.1	25.3	5.8	4.1	78.2	56.6	19.6	14.1	89.45	64.5	149.1	0.45
18	11	146.3	37.5	25.6	5.5	3.8	80.8	61.4	13.5	9.2	97.63	66.9	163.2	0.42
19	12	164.9	42.1	25.5	7.3	4.4	96.3	58.4	19.2	11.7	107.72	65.3	179.5	0.43

TABLE III.
Carotenoid pigments of Desi variety mangoes during different stages of ripening on storage at room temperature.

Serial number.	Number of days after plucking.	Total pigments, $\mu\text{g./g.}$	Xanthophyll, $\mu\text{g./g. percent.}$	Neo-xanthophyll, $\mu\text{g./g. percent.}$	β -carotene, $\mu\text{g./g. percent.}$	Neo- β -carotene B, $\mu\text{g./g. percent.}$	Total active pigments in terms of β -carotene, $\mu\text{g./g. percent.}$	Vitamin A potency, I.U. per g.	Xanthophyll: carotene ratio.
1	1	7.5	4.7	0.7	1.7	0.4	1.00	3.2	2.76
2	3	10.3	6.3	1.0	2.5	0.5	2.75	4.6	2.52
3	3	11.0	6.5	1.2	2.6	0.7	2.95	4.9	2.50
4	4	12.1	7.0	1.3	3.0	0.8	3.40	5.7	2.33
5	4	12.5	7.2	1.4	3.1	0.8	3.50	5.8	2.32
6	5	14.3	8.2	1.5	3.7	0.9	4.15	6.9	2.21
7	5	15.4	8.9	1.5	4.0	1.0	4.50	7.5	2.22
8	6	16.8	9.7	1.6	4.1	1.4	4.80	8.0	2.36
9	6	17.7	9.8	1.9	4.5	1.5	5.25	8.8	2.17
10	7	19.2	10.0	2.9	5.0	1.3	5.65	9.4	2.00
11	7	19.8	10.2	3.0	5.3	1.3	5.95	9.9	1.92
12	10	22.9	10.6	4.0	6.7	1.6	7.50	12.5	1.58
13	10	24.4	11.2	4.3	7.1	1.8	8.00	13.3	1.58
14	11	26.8	13.1	4.5	7.1	1.8	8.00	13.3	1.80
15	11	31.1	17.3	4.7	9.8	2.3	10.95	18.3	1.77
16	12	38.1	17.1	4.8	12.1	4.1	14.15	23.6	1.44

TABLE IV.
Carotenoid pigments of Chounsa mangoes during different stages of ripening at room temperature.

Serial number.	Number of days after plucking.	Total pigments. $\mu\text{g./g.}$	Xanthophyll. $\mu\text{g./g. per cent.}$	Neo-xanthophyll. $\mu\text{g./g. per cent.}$	β -carotene. $\mu\text{g./g. per cent.}$	Neo- β -carotene B. $\mu\text{g./g. per cent.}$	Total active pigments in terms of β -carotene. $\mu\text{g./g. per cent.}$	Vitamin A potency. I.U. per g.	Xanthophyll: carotene ratio.
1	..	1.4	0.8	0.4	0.2	..	0.20	0.3	4.00
2	1	1.9	1.1	0.5	0.3	..	0.3	0.5	3.66
3	1	2.4	1.2	0.3	0.9	..	0.9	1.5	1.33
4	2	5.4	2.4	1.3	1.7	..	1.7	2.8	1.41
5	2	7.9	3.5	1.9	2.5	..	2.5	4.2	1.40
6	6	8.0	3.5	2.0	2.5	..	2.5	4.2	1.40
7	6	8.2	3.6	1.2	2.7	0.7	3.05	5.1	1.33
8	7	9.9	4.4	1.8	3.7	..	3.7	6.2	1.19
9	7	9.6	3.8	1.6	4.2	..	4.2	7.0	0.90
10	8	9.4	4.0	0.9	4.5	..	4.5	7.5	0.88
11	8	10.9	4.6	0.8	4.0	1.5	4.75	7.9	1.15
12	9	16.5	6.8	1.1	6.3	2.3	7.15	12.4	1.08
13	11	24.1	9.7	0.9	10.5	3.0	12.00	20.0	0.92

TABLE V.
Carotenoid pigments of Chounsa mangoes during different stages of ripening on storage at 42°C.

Serial number.	Number of days after plucking.	Total pigments. $\mu\text{g./g.}$	Xanthophyll. $\mu\text{g./g. per cent.}$	Neo-xanthophyll. $\mu\text{g./g. per cent.}$	β -carotene. $\mu\text{g./g. per cent.}$	Neo- β -carotene B. $\mu\text{g./g. per cent.}$	Total active pigments in terms of β -carotene. $\mu\text{g./g. per cent.}$	Vitamin A potency. I.U. per g.	Xanthophyll: carotene ratio.
1	1	3.0	1.8	60.0	0.6	20.0	0.6	20.0	3.00
2	1	4.3	2.3	53.5	0.6	14.0	1.4	32.5	1.61
3	2	4.6	2.0	43.5	1.2	26.1	1.4	30.4	1.43
4	2	5.5	2.4	44.6	1.3	23.6	1.8	32.7	1.33
5	4	7.0	2.6	37.1	2.5	35.7	1.9	27.1	1.37
6	6	6.9	3.3	47.7	0.9	13.1	2.7	39.1	1.22
7	6	10.0	4.5	45.0	1.1	11.0	3.6	36.0	1.25
8	7	14.2	6.2	43.7	2.4	16.9	5.6	39.4	1.11
9	7	22.9	12.2	53.3	1.1	4.8	7.3	31.9	1.67
10	11	38.7	16.3	42.1	2.3	5.9	14.8	38.2	1.10

DISCUSSION AND CONCLUSIONS.

The results of chromatographic separation and estimation of the carotenoid pigments of *Saroli*, *Desi* and *Chounsa* mangoes, summarized in Tables I to V, show that all the carotenoids increase during ripening. The change in the colour of the pulp from pale yellow to rich reddish-yellow is a fair indication of the increase in carotenoid content.

In the case of first lot of *Saroli* mangoes plucked when these were still growing in size, it is found that the fruit attains the maximum concentration of carotenoids in about 15 days, whereas those plucked after attaining the mature-green stage, the maximum concentration of carotenoids is reached in 12 days. The increase in the amount of the different carotenoids is almost of the same order in both lots of *Saroli* mangoes examined. The concentration of neo- β -carotene B, an isomer of β -carotene, is found to be principally a function of the β -carotene concentration. Usually one $\mu\text{g.}$ of neo- β -carotene B is present to every 5 $\mu\text{g.}$ to 7 $\mu\text{g.}$ of β -carotene. Neo- β -carotene U, another isomer of β -carotene, too is a function of β -carotene concentration. In general, about 10 to 15 per cent of the β -carotene fraction obtained after chromatographic separation is neo- β -carotene U.

The amount of β -carotene increases from 3.9 $\mu\text{g./g.}$ when unripe to 96.3 $\mu\text{g./g.}$ when it was completely ripe. There was also a steady increase in the amount of the two isomers of β -carotene, neo- β -carotene B and neo- β -carotene U; as the fruit ripens, the increase being 0.8 to 19.2 $\mu\text{g./g.}$ in the case of neo- β -carotene B and 1.1 to 7.3 $\mu\text{g./g.}$ in the case of neo- β -carotene U. Unlike tomatoes and leafy vegetables, the amount of xanthophyll also increases steadily as the fruit ripens, the increase being 6.7 to 42.1 $\mu\text{g./g.}$ Vitamin A potency had increased from 7.5 to 161.5 I.U./g. in the case of first lot of *Saroli* mangoes, and from 7.6 to 179.5 I.U./g. in the case of second lot of this variety examined.

Desi mangoes also showed a similar steady increase in the amount of all the carotenoid pigments as the fruit ripens though the amount of total as well as individual carotenoids is much less as compared to *Saroli* mangoes. In this variety of fruit, neo- β -carotene U could not be detected but an additional band of neo-xanthophyll was found to be present.

The amount of β -carotene increased from 1.7 to 12.1 $\mu\text{g./g.}$, while that of neo- β -carotene B from 0.4 to 4.1 $\mu\text{g./g.}$ In this variety, however, the amount of xanthophyll increased to a greater degree than β -carotene as compared to *Saroli* mangoes, the increase being from 4.7 to 17.4 $\mu\text{g./g.}$

The amount of vitamin A potency increased from 3.2 to 23.6 I.U. per g. during this interval.

In the case of *Chounsa* mangoes, it is found that mangoes ripened at 42°C. in 11 days are higher in total as well in individual carotenoids than those ripened at room temperature for 11 days. This is probably due to the fact that pigments are synthesized at a greater speed in mangoes ripened at 42°C. as compared to mangoes ripened at room temperature. The amount of β -carotene increased from 0.2 to 10.5 $\mu\text{g./g.}$ in 11 days in mangoes ripened at room temperature, while those ripened at 42°C. increased from 0.6 to 14.8 $\mu\text{g./g.}$ in 11 days. During this interval, xanthophyll showed an increase from 1.8 to 16.3 $\mu\text{g./g.}$ in mangoes ripened at 42°C. as compared to 0.8 to 9.7 $\mu\text{g./g.}$ in mangoes ripened at room temperature.

The vitamin A potency increased from 0.3 to 20.0 I.U. per g. in the case of mangoes ripened at room temperature, while from 1.0 to 29.1 I.U. per g. in the case of mangoes ripened at 42°C.

It is found that, while all the pigments are synthesized during ripening, β -carotene increases at a greater rate than any other pigment. The ratio of xanthophyll to β -carotene in the unripe mango gradually decreases during the process of ripening. The synthesis of β -carotene proceed at the rapid rate of 5.0 to 10.0 $\mu\text{g./g.}$ of tissue in 24 hours. Thus, an average-sized mango may synthesize as much as 1,200 $\mu\text{g.}$ of β -carotene in a day. The rate of synthesis, of course, varies in different varieties within a certain range.

SUMMARY.

The changes occurring in the individual carotenoid pigments of three varieties of mango fruit during different stages of ripening are reported in this paper. It has been observed that all the carotenoid pigments increase during ripening, β -carotene increasing at a greater rate than any other pigment. Unlike tomatoes and leafy vegetables, xanthophyll also shows a similar gradual increase as the fruit ripens. An average-sized mango may synthesize 1,200 $\mu\text{g.}$ of β -carotene in 24 hours.

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THE STABILITY OF ADDED CAROTENE IN VANASPATHI UNDER DIFFERENT CONDITIONS OF STORAGE.

BY

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THE fortification of staple foodstuffs has been undertaken extensively in Europe and is now well recognized as a means of producing adequate nutritional factors to the population on a large scale. Vitamin A is already deficient in the diet of the Indian people. Since ghee is becoming scarce in the country and more and more of hydrogenated fats are entering into the dietary of the population, the fortification of edible fats with vitamin A is a matter of considerable importance.

Carotene is found widely distributed in nature and in some instances, notably carrot, in sufficient concentration and availability to warrant the development of processes for its commercial isolation. Since vitamin A and carotene are subject to destruction, it is essential that they should not be added to foods in which they are not relatively stable or the destruction be inhibited to the fullest degree. Evidence to date has established that tocopherols and allied substances in association with phosphatidic materials are largely responsible for the stability exhibited by natural vegetable oils. Olcott and Emmerson (1941) showed that tocopherols are increasingly effective as anti-oxidants in lards in the order *alpha*, *beta* and *gamma*; *gamma*-form having three times the anti-oxygenic index of *alpha*-tocopherol and the activity of *beta*-tocopherol being intermediate between the other two. Esters of tocopherols were, however, ineffective as anti-oxidants. Later, Bird (1941), Sullmann (1941) and Quackenbush, Cox and Steenbock (1942) have shown *alpha*-tocopherol to be an effective anti-oxidant for vitamin A and carotene.

Hume and Smedley-Maclean (1930), Olcovich and Mattill (1931) and Morgal, Byers and Miller (1943) reported hydroquinone at 0.01 and 0.1 per cent levels to be very effective in retarding carotene decomposition. Broadway and Mattill (1934) reported that crude carotene is usually accompanied by natural anti-oxidants and this is true of carrots. Olcott and Mattill (1936) pointed out that many leafy green vegetables contain vitamin E which are concentrated along with the carotene and sterols in the unsaponifiable fraction. Therefore, to compare the stability of carotene when incorporated in vanaspati, investigations reported in this paper were undertaken to determine the relation between the rate and extent of destruction, with or without the addition of anti-oxidants and when kept under various conditions. This will bring to light any similarity between the action of added anti-oxidants used and of those naturally occurring in carrots.

CAROTENIZED GHEE.

The carotenized ghee was prepared by adding de-hydrated carrots to hot vanaspati followed by decantation and filtration.

EXPERIMENTAL.

The ghee was put into twelve 4-oz. glass-bottles and six ordinary cigarette tins. These bottles and tins were then sealed with bees-wax and kept under the following different conditions:—

- (1) At room temperature, in light, in a glass-bottle.
- (2) At room temperature, in dark, in a glass-bottle.
- (3) At room temperature, in a tin.
- (4) At room temperature, in light, with α -tocopherol, in a glass-bottle.
- (5) At room temperature, in dark, with α -tocopherol, in a glass-bottle.
- (6) At room temperature, in light, with ethyl gallate, in a glass-bottle.
- (7) At room temperature, in dark, with ethyl gallate, in a glass-bottle.
- (8) At room temperature, with α -tocopherol, in a tin.
- (9) At room temperature, with ethyl gallate, in a tin.
- (10) In refrigerator, in light, in a glass-bottle.
- (11) In refrigerator, in dark, in a glass-bottle.
- (12) In refrigerator, in a tin.
- (13) In refrigerator, in light, with α -tocopherol, in a glass-bottle.
- (14) In refrigerator, in dark, with α -tocopherol, in a glass-bottle.
- (15) In refrigerator, in light, with ethyl gallate, in a glass-bottle.
- (16) In refrigerator, in dark, with ethyl gallate, in a glass-bottle.
- (17) In refrigerator, with α -tocopherol, in a tin.
- (18) In refrigerator, with ethyl gallate, in a tin.

The anti-oxidants, α -tocopherol and ethyl gallate, were added at 0.1 per cent level based on the weight of the oil.

TABLE I.
Stability of added carotene in vanaspai during storage at room temperature.

AT ROOM TEMPERATURE, IN LIGHT, IN A GLASS-BOTTLE.				AT ROOM TEMPERATURE, IN DARK, IN A GLASS-BOTTLE.				AT ROOM TEMPERATURE, IN A TIN.			
Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.
1	...	8.47	...	1	...	8.47	...	1	...	8.47	...
2	7	8.33	1.6	2	7	8.40	0.8	2	8	8.30	2.0
3	14	7.95	6.1	3	14	8.00	5.5	3	14	7.67	9.4
4	21	7.48	11.7	4	21	7.58	10.5	4	21	7.32	13.4
5	37	7.35	13.2	5	37	7.45	12.0	5	37	7.17	15.3
6	42	7.04	16.8	6	42	7.14	15.7	6	42	7.00	17.3
7	49	6.87	18.8	7	49	7.00	17.3	7	49	6.79	19.7
8	58	6.35	25.0	8	58	6.45	23.8	8	58	6.27	25.9
9	63	6.22	26.5	9	63	6.27	25.8	9	64	6.17	27.1
10	81	6.14	27.3	10	81	6.11	27.7	10	79	6.00	29.1
11	135	5.87	30.6	11	135	6.00	29.1	11	136	5.80	30.6

TABLE I—*contd.*
Stability of added carotene in vanaspati during storage at room temperature.

AT ROOM TEMPERATURE, IN LIGHT, WITH α -TOCOPHEROL, IN A GLASS-BOTTLE.					AT ROOM TEMPERATURE, IN DARK, WITH α -TOCOPHEROL, IN A GLASS-BOTTLE.					AT ROOM TEMPERATURE, IN LIGHT, WITH ETHYL GALLATE, IN A GLASS-BOTTLE.				
Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.			
1	...	8.47	...	1	...	8.47	...	1			
2	2	2			
3	3	3			
4	22	8.14	3.8	4	22	8.10	4.3	4	22	7.94	6.2			
5	37	7.78	8.1	5	38	7.70	9.1	5	37	7.50	11.4			
6	42	7.68	9.3	6	44	7.60	10.3	6	43	7.26	14.3			
7	49	7.46	11.9	7	51	7.33	13.4	7	50	7.00	17.3			
8	59	6.86	19.0	8	59	6.99	17.2	8	59	6.73	20.5			
9	64	6.75	20.3	9	64	6.51	23.1	9	65	6.56	22.5			
10	79	6.72	20.6	10	79	6.49	23.4	10	79	6.26	26.1			
11	135	6.14	27.5	11	136	6.14	27.2	11	136	6.00	29.1			

TABLE I—*concl.*
Stability of added carotene in vanaspathi during storage at room temperature.

AT ROOM TEMPERATURE, IN DARK, WITH ETHYL GALLATE, IN A GLASS-BOTTLE.				AT ROOM TEMPERATURE, WITH α -TOCOPHEROL, IN A TIN.				AT ROOM TEMPERATURE, WITH ETHYL GALLATE, IN A TIN.			
Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.
1	...	8.47	...	1	...	8.47	...	1	...	8.47	...
2	2	2
3	3	3
4	22	8.00	5.5	4	4
5	38	7.57	10.6	5	38	7.69	9.2	5	39	7.57	10.6
6	44	7.30	13.8	6	44	7.35	13.2	6	44	7.29	13.9
7	51	7.03	17.0	7	51	7.23	14.6	7	51	7.00	17.3
8	59	6.60	22.1	8	59	6.76	20.2	8	59	6.58	22.3
9	61	6.48	23.5	9	61	6.62	21.8	9	64	6.49	23.4
10	80	6.30	25.6	10	80	6.27	26.0	10	80	6.28	25.8
11	141	6.09	28.1	11	142	6.14	27.5	11	141	6.02	28.9

TABLE II.
Stability of added carotene in vanaspathi during storage in refrigerator.

IN LIGHT, IN A GLASS-BOTTLE.				IN DARK, IN A GLASS-BOTTLE.				IN A TIN.			
Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.
1	0	8.47	...	1	0	8.47	...	1	0	8.47	...
2	2	8	8.26	2.4	2	8	8.33	1.6
3	15	8.29	2.1	3	15	8.16	3.6	3	15	8.18	3.4
4	21	8.00	5.5	4	21	8.02	5.3	4	21	7.81	7.8
5	38	7.67	9.4	5	38	7.72	8.9	5	38	7.56	10.7
6	43	7.39	12.7	6	43	7.42	12.3	6	43	7.15	15.6
7	50	7.10	16.1	7	50	7.12	15.9	7	50	6.98	17.6
8	59	7.01	17.2	8	60	6.78	19.9	8	60	6.83	19.3
9	65	6.55	22.6	9	9	65	6.80	19.7
10	80	6.23	26.4	10	80	6.31	25.5	10	80	6.20	26.8
11	142	6.01	29.0	11	142	6.11	27.8	11	142	6.12	27.8

TABLE II—contd.
Stability of added carotene in vanaspathi during storage in refrigerator.

IN LIGHT, WITH α -TOCOPHEROL, IN A GLASS-BOTTLE.				IN DARK, WITH α -TOCOPHEROL IN A GLASS-BOTTLE.				IN LIGHT, WITH ETHYL GALLATE, IN A GLASS-BOTTLE.			
Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.
1	0	8.47	...	1	0	8.47	...	1	0	8.47	...
2	2	2
3	3	3
4	28	8.22	3.0	4	23	8.27	2.3	4	23	8.12	4.1
5	39	8.00	5.5	5	39	8.07	4.7	5	39	7.77	8.2
6	45	7.95	6.1	6	45	7.95	6.1	6	45	7.48	11.7
7	53	7.56	10.7	7	52	7.72	8.9	7	53	7.29	13.9
8	60	7.39	12.7	8	63	7.68	9.3	8	60	6.89	18.6
9	65	6.91	18.4	9	66	7.44	12.1	9	66	6.65	21.5
10	82	6.88	18.7	10	81	6.98	17.6	10	81	6.60	22.1
11	143	6.50	23.2	11	143	6.58	22.3	11	139	6.19	26.9.

TABLE II—concl'd.
Stability of added carotene in vanaspathi during storage in refrigerator.

IN DARK, WITH ETHYL GALLATE, IN A GLASS-BOTTLE.				WITH α -TOCOPHEROL, IN A TIN.				WITH ETHYL GALLATE, IN A TIN.			
Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.	Number.	Storage time in days.	β -carotene. $\mu\text{g./g.}$	Loss, per cent.
1	0	8.47	...	1	0	8.47	...	1	0	8.47	...
2	2	2
3	3	3
4	23	8.15	3.7	4	24	8.22	3.0	4	24	8.19	3.3
5	39	7.85	7.3	5	39	7.91	6.6	5	38	7.80	8.0
6	45	7.54	10.9	6	46	7.87	7.1	6	46	7.71	9.0
7	52	7.45	12.0	7	52	7.45	12.0	7	53	7.48	11.7
8	63	7.14	15.7	8	63	7.32	13.5	8	63	7.39	13.3
9	66	6.99	17.5	9	66	6.90	18.5	9	66	7.00	17.3
10	82	6.69	21.0	10	82	6.84	19.2	10	81	6.63	21.7
11	142	6.21	26.7	11	143	6.40	24.4	11	143	6.18	27.0

METHOD OF ANALYSIS.

A 10-g. sample of accurately weighed ghee was transferred to a 500 c.c. digestion flask and 200 c.c. of 30 per cent ethanolic potassium hydroxide added to it. It was then refluxed over a water-bath for 45 minutes while still hot, 200 c.c. of water were added to it and allowed to cool under water. The ethanolic water-solution was transferred to a separatory funnel and about 150 c.c. of petroleum ether were added to it and shaken thoroughly. After complete separation of the two layers, the alcoholic solution was drawn off and a fresh portion of petroleum ether was added. This process was continued until no more pigment was extracted into the petroleum ether layer. The combined petroleum ether fractions were washed with water until last traces of alkali and alcohol were removed. The combined extracts were dried over anhydrous sodium sulphate, concentrated to a small volume under reduced pressure in an atmosphere of nitrogen. The concentrated solution was finally drawn through an aluminium-oxide adsorption column, and washed with a little petroleum ether containing 20 per cent benzene. A layer of anhydrous sodium sulphate (0.5 cm.) was placed above the adsorbant and the column was washed with a little petroleum ether before the extract was poured in. The column was pushed out and β -carotene band eluted with 3 per cent ethanol-petroleum ether mixture. The eluate was made up to volume and its amount estimated calorimetrically using 0.04 per cent $K_2Cr_2O_7$ as the standard. The results are expressed as $\mu\text{g./g.}$

Stability tests were run on the same samples for about 135 days. At appropriate intervals, samples were taken from different bottles and tins and analysed for β -carotene content. The values for individual determinations for the percentage loss in β -carotene at room temperature and in the refrigerator for the various samples are recorded in Tables I and II.

DISCUSSION.

The data in Tables I and II show that both α -tocopherol and ethyl gallate at 0.1 per cent level are ineffective as anti-oxidants for carotene stability. The ineffectiveness of the added anti-oxidants is presumably because the carotenized ghee already contained sufficient amount of anti-oxidants which are extracted along with carotene so that addition of α -tocopherol and ethyl gallate, in the quantities used, did not practically add to the carotene stability.

SUMMARY.

Carotene derived from carrots contained sufficient amount of naturally occurring anti-oxidants and little protection was observed by additional anti-oxidants at 0.1 per cent level.

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MUTUAL INFLUENCE OF MINERALS IN METABOLISM.

BY

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INTRODUCTION.

THE essential nature of the elements, such as calcium, phosphorus, magnesium, iron, copper, manganese and others, for the proper nutrition of the human body has long been established and their requirements have been investigated by different workers. These elements are not taken singly but are taken together with our daily diet. It is not improbable that the elements may have some mutual influence in their metabolic behaviour.

The present investigation was, therefore, undertaken to make a systematic study of the mutual effect of the above elements in metabolism by direct balance experiments on human subjects.

The problem assumes considerable importance in view of the fact that large doses of calcium salts are ingested in the treatment of rickets, osteomalacia, etc., and large doses of iron salts are administered in the treatment of anæmia and it is not unlikely that the excess intake of calcium, iron and other salts may influence the metabolism of the others to a considerable extent.

EXPERIMENTAL.

The experiments were conducted on six normal adults, their ages varying from 24 to 28 years and their weights from 45 to 50 kilos. The composition of the basal diets is indicated in Table I. In each series of the experiments the cereals and pulses were taken from the same stock every day and aliquots of vegetables and fish taken daily were pooled for analysis. With each particular experiment the experimental subjects were daily given carefully weighed diets which they consumed *in toto* in two portions.

TABLE I.

Composition of the diets.

(The figures are expressed in grammes.)

Name of the foodstuff.	Rice-fish diet. (D I).	Vegetarian rice diet. (D II).	Vegetarian whole-wheat diet. (D III).
Rice	550-600	600	...
Whole-wheat	500-600
Pulse	60	100	70-90
Fish	70
Vegetables :—			
Potato	100	100	100
Brinjal	*100	*100	*100
Bean			
Patól			
Pumpkin-gourd			
Sugar	50
Mustard oil	30	30	...
Ghee (butter-fat)	30

* For any particular experiment any two of the four items each weighing 50 g. were selected.

The subjects were fed on the basal diets for six days and supplementation with calcium, magnesium, iron and manganese salts was continued for a further period of 3 to 6 days. Each period included 3 consecutive days and the chronological order of the experimental periods is denoted by the arithmetical numbers; period I, which does not figure in the table, must be assumed to be the preliminary period. The technique of the experiment was the same as reported in our previous communication (Basu, Basak and De, 1941) from this Laboratory.

Special precautions were taken to avoid the possibility of iron, copper and manganese contamination. Aluminium vessels were used for the purpose of cooking of the diets and collecting and drying of the faeces. The analysis of the above elements was carried out with re-distilled water in a specially prepared glass-room of the Laboratory.

The following methods were adopted for the analysis of foodstuffs, urine, and faeces :—

CALCIUM AND PHOSPHORUS : 'Practical Physiological Chemistry,'
by Hawk and Bergeim (1938).

MAGNESIUM : With 8-hydroxyquinolin reagent by the method of Greenberg and Mackey (1932) as modified by Hummel *et al.* (1936).

IRON : By the method of Farrar (1935).

COPPER : With 2 : 4 dithiocarbamate reagent by the method of Callan and Henderson (1929) as modified by Hoar (1937).

MANGANESE : With potassium periodate reagent by the method of Skinner and Peterson (1930).

RESULTS.

A.—Effect of calcium on the metabolism of magnesium, phosphorus, iron, copper and manganese.—Calcium-iron antagonism was first suggested by Rose and Vahlteich (1932) and their view was further supported by the work of Shelling and Josheps (1934) and Kletzein (1940) done on rats and other laboratory animals.

The disease of perosis in chicks and turkeys was previously regarded as due to dietary deficiency of manganese, and the observation of Wilgus, Norris and Heuser (1937), Wilgus and Patton (1939) and others that high calcium in the diet aggravates the disease led to the assumption that calcium affects manganese utilization. Hammond (1936) placed the responsibility upon the excess of phosphorus in the diet, while Schaible, Bendermer and Davidson (1938) pointed out that both calcium and phosphorus in high doses are responsible for increasing the incidence of perosis. The work of the above investigators in the study of the causative factors for the development of perosis has, however, indicated a mutual antagonism between calcium and manganese, and phosphorus and manganese in metabolism.

The mutual effect of calcium and magnesium in metabolism has also been studied in different laboratories but the results obtained show great discrepancy. In contradiction to the observations of Malcolm (1905) that high calcium in the diet does not affect magnesium excretion, Heller and Haddard (1936) and Tufts and Greenberg (1938) showed that high calcium intake reduces magnesium retention and increases the severity of its deficiency.

To get more precise information about the rôle of calcium on the utilization of other nutritionally essential elements such as magnesium, phosphorus, iron, copper and manganese, some balance experiments have been performed by administering high doses of different calcium salts along with the basal diets. The results presented in Table III show that calcium ingested in the form of lactate, gluconate, oxide and milk decreases the retention of magnesium, iron, copper and manganese to an appreciable extent. The effect on phosphorus metabolism was studied by ingestion of calcium in the form of lactate and the results obtained (*see* Table II) show that high dose of calcium salt is also detrimental to phosphorus metabolism.

TABLE II.

Showing the effect of calcium as lactate on the metabolism of phosphorus.
(The figures indicate the daily average values in mg.)

Experimental subject.	Diet.	Supplement.	Period.	PHOSPHORUS METABOLISM.			
				Dietary. P	Urinary. P	Fæcal. P	Balance.
G. C. D. ...	D II	Nil. 500 mg. Ca as lactate.	P II Average of P III and IV.	1,048 1,048	398 417	502 561	+148 + 70
G. C. N. ...	D I	Nil. 1,000 mg. Ca as lactate.	P II Average of P III and IV.	1,290 1,290	402 418	584 663	+304 +209

TABLE III.

(A)—Effect of calcium as gluconate and lactate on the metabolism of magnesium and iron.
(The figures indicate the daily average values in mg.)

Experimental subject.	Diet.	Supplement.	Period.	MAGNESIUM METABOLISM.				IRON METABOLISM.			
				Dietary Mg.	Urinary Mg.	Faecal Mg.	Balance.	Dietary Fe.	Urinary Fe.	Faecal Fe.	Balance.
G. C. N. ...	D I ...	Nil. 1,000 mg. Ca as lactate.	P II Average of P III and IV.	432.1 432.1	56.1 68.9	243.4 299.4	+ 132.0 + 63.8	33.18 33.18	0.47 0.55	25.83 45.43	+ 6.88 - 12.8
G. C. D. ...	D I ...	Nil. 500 mg. Ca as lactate.	P II Average of P III and IV.	394.6 394.6	25.2 45.8	141.2 241.0	+ 228.2 + 107.8	38.42 38.42	0.39 0.42	32.80 46.96	+ 5.23 - 8.96
P. C. D. ...	D II ...	Nil. 300 mg. Ca as gluconate.	P II Average of P III and IV.	355.4 355.4	45.4 56.3	150.3 189.2	+ 159.7 + 109.9

(B)—Effect of calcium of milk.

P. C. G. ...	D II ...	Nil. 357 mg. Ca from 270 c.c. milk.	P II Average of P III and IV.	301.0 326.0*	71.0 61.2	147.7 252.2	+ 82.3 + 12.6	26.74 26.88*	0.68 0.73	16.66 24.5	+ 9.40 + 1.65
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* The extra magnesium and iron were available from the milk supplement.

TABLE III—*contd.*

(A)—*Effect of calcium as gluconate and lactate on the metabolism of copper and manganese.*
 (The figures indicate the daily average values in mg.)

Experimental subject.	Diet.	Supplement.	Period.	COPPER METABOLISM.				MANGANESE METABOLISM.		
				Dietary Cu.	Urinary Cu.	Faecal Cu.	Balance.	Dietary Mn.	Faecal Mn.	Balance.
G. C. N. ...	D I ...	Nil. 1,000 mg. Ca as lactate.	P II Average of P III and IV.	4.08	0.34	1.89	+1.85	10.98	6.54	+4.44
				4.08	0.31	3.70	+0.07	10.98	14.48	-3.50
G. C. D. ...	D I ...	Nil. 500 mg. Ca as lactate.	P II Average of P III and IV.	2.38	0.29	1.83	+0.26	9.48	5.86	+3.62
				2.38	0.30	2.02	-0.56	9.48	10.05	-0.57
P. C. D. ...	D II ...	Nil. 300 mg. Ca as gluconate.	P II Average of P III and IV.	8.07	4.77	+3.30
				8.07	6.60	+1.47
(B)— <i>Effect of calcium of milk.</i>										
P. C. G. ...	D II ...	Nil. 357 mg. Ca 270 c.c. milk.	P II Average of P III and IV.	4.06	0.39	2.16	+1.51	7.59	6.45	+1.14
				4.08*	0.26	3.08	+0.74	7.81*	7.48	+0.38

* The extra copper and manganese were available from the milk supplement.

TABLE III—*contd.*

(C)—*Effect of calcium as oxide ingested with betel leaves and nut on the metabolism of magnesium and iron.*

(The figures indicate the daily average values in mg.)

Experimental subject.	Diet.	Supplement.	Period.	MAGNESIUM METABOLISM.				IRON METABOLISM.			
				Dietary Mg.	Urinary Mg.	Faecal Mg.	Balance.	Dietary Fe.	Urinary Fe.	Faecal Fe.	Balance.
G. C. D. ...	D III ...	Nil. 415 mg. Ca as oxide.	P II Average of P III and IV.	473.8 490.2*	69.1 23.6	181.6 312.5	+ 224.1 + 154.1	43.92 44.34*	0.46 0.49	36.9 51.2	+ 6.56 - 7.35
P. C. G. ...	D III ...	Nil. 397 mg. Ca as oxide.	P II Average of P III and IV.	493.4 516.1*	34.1 54.9	269.7 312.6	+ 189.6 + 148.6	45.82 46.23*	0.48 0.46	37.41 52.60	+ 7.93 - 6.83
P. C. D. ...	D I ...	Nil. 331 mg. Ca as oxide.	P II Average of P III and IV.	351.4 370.8*	54.8 67.8	192.2 221.6	+ 104.4 + 81.4	39.81 40.03*	0.63 0.84	28.12 36.61	+ 11.06 + 2.58
S. G. P. ...	D I ...	Nil. 383 mg. Ca as oxide.	P II Average of P III and IV.	324.2 343.8*	47.2 56.6	163.9 211.7	+ 113.1 + 75.5

* The extra magnesium and iron were available from the betel leaves and nut with which the calcium oxide was ingested.

TABLE III—*concl'd.*
 (C)—Effect of calcium as oxide ingested with betel leaves and nut on the metabolism
 of copper and manganese.

(The figures indicate the daily average values in mg.)

Experimental subject.	Diet.	Supplement.	Period.	COPPER METABOLISM.				MANGANESE METABOLISM.		
				Dietary Cu.	Urinary Cu.	Faecal Cu.	Balance.	Dietary Mn.	Faecal Mn.	Balance.
G. C. D. ...	D III ...	Nil. 415 mg. Ca as oxide.	P II Average of P III and IV.	7.18	0.31	3.20	+ 3.67	24.58	16.27	+ 8.31
				7.24*	0.36	3.91	+ 2.97	24.60*	23.14	+ 1.46
P. C. G. ...	D III ...	Nil. 397 mg. Ca as oxide.	P II Average of P III and IV.	6.45	0.32	4.15	+ 1.98	17.25	14.79	+ 2.46
				6.49*	0.37	4.93	+ 1.19	17.27*	17.42	— 0.15
P. C. D. ...	D I ...	Nil. 331 mg. Ca as oxide.	P II Average of P III and IV.	6.28	0.41	4.15	+ 1.72	12.43	8.61	+ 3.82
				6.32*	0.48	5.24	+ 0.60	12.46*	10.96	+ 1.50
S. G. P. ...	D I ...	Nil. 383 mg. Ca as oxide.	P II Average of P III and IV.	8.89	5.97	+ 2.92
				8.92*	7.45	+ 1.47

* The extra copper and manganese were available from the betel leaves and nut with which the calcium oxide was ingested.

B.—Effect of magnesium on the utilization of calcium, phosphorus, iron, copper and manganese.—There is much controversy regarding the effect of high magnesium intake on the utilization of calcium and phosphorus. A group of workers such as Barbour and Winter (1931), Euler and Rydbom (1931) and others showed that magnesium could cure rickets and osteomalacia and bring about improved calcium retention. Another group of workers, as Buckner, Martin and Insko (1932) and Cunningham (1933), on the contrary, observed detrimental effect of magnesium on bone formation and calcium utilization.

As regards its effect on the utilization of other nutritionally essential elements such as iron, copper and manganese, no information is available in the literature. Some human metabolic studies have, therefore, been performed by administering high dose of magnesium salt with the diet to get a clear picture of the above subject of nutritional importance. The results of the experiment are shown in Table IV from which it will be observed that the administration of 500 mg. of magnesium as oxide with the basal diets decreases the retention of calcium, phosphorus, iron, copper and manganese to a considerable extent. Since magnesium like calcium was found to possess similar antagonistic effect on the metabolism of other nutritionally essential elements, the view of Duckworth (1938-39)—‘Although chemical similarities exist between calcium and magnesium, it would be unwise to assume that under any given conditions, their behaviour in metabolism will be similar. There seems to be no reason to believe that the elements have nearly similar metabolic path’—does not seem to be correct. In the light of the present observations it may be suggested that strontium, beryllium and other elements possessing similar chemical properties as those of calcium should also behave alike in metabolism. The production of rickets in animals after ingestion of the above elements as observed by Sobel *et al.* (1934, 1935, 1936) and others is most probably due to their antagonistic effect on the metabolism of calcium and phosphorus.

C.—The effect of iron on the utilization of calcium, phosphorus, magnesium, copper and manganese.—Iron-calcium antagonism had been demonstrated by Brock and Diamond (1934), Deobald and Elvehjem (1935) and others who by incorporating a large dose of iron salt in the diets of the rats and chicks, observed severe rickets and lowering of bone-ash in these animals. Barer and Fowler (1940), however, in their experiments on sixteen females did not find any effect of administration of medicinal iron on the utilization of calcium and phosphorus. Some balance experiments have, therefore, been performed with high dose of iron salt in the diet to have clear information about the relation of iron to other elements in metabolism. It is evident from the results presented in Table V that the administration of 100 mg. of iron as ferrous ammonium sulphate and nearly 75 mg. in the form of ‘Fersolate’—a medicinal preparation of Glaxo Laboratories—decreases the utilization of calcium, magnesium, phosphorus, copper and manganese to a significant extent. The results of the present investigation are in contradiction to those of Barer *et al.* (*loc. cit.*) but in good agreement with those of Rehm and Winters (1940) who observed decreased utilization of calcium and phosphorus in rats due to addition of ferric chloride to the basal diet. Rickets in animals as observed by Cox *et al.* (1931) and other workers by ingesting high dose of iron salt with the basal diet is probably due to interference of the added dose in the utilization of calcium and phosphorus.

TABLE IV.

Showing the effect of magnesium oxide on the metabolism of calcium, phosphorus, iron, copper and manganese.

(The figures indicate the daily average values in mg.)

Experimental subject.	Diet.	Supplement.	Period.	CALCIUM METABOLISM.				PHOSPHORUS METABOLISM.			
				Dietary Ca.	Urinary Ca.	Faecal Ca.	Balance.	Dietary P.	Urinary P.	Faecal P.	Balance.
P. C. G. ...	D II ...	Nil.	P II	258.4	13.0	409.6	- 164.2	998	304	588	+ 106
		500 mg. magnesium as oxide.	Average of P III and IV.	258.4	37.6	475.3	- 254.5	998	332	619	+ 47
G. C. D. ...	D I ...	Nil.	P II	558.4*	49.8	364.2	+ 154.4	1,151	441	596	+ 114
		500 mg. magnesium as oxide.	Average of P III and IV.	558.4	62.3	492.1	+ 4.0	1,151	501	633	+ 17

* The high calcium content of this rice diet is due to incorporation of small fish in the diet, which was consumed *in toto*.

TABLE VI.

Showing the effect of manganese as chloride on the metabolism of calcium, magnesium, phosphorus, iron and copper.

(The figures indicate the daily average values in mg.)

Experimental subject.	Diet.	Supplement.	Period.	CALCIUM METABOLISM.				MAGNESIUM METABOLISM.			
				Dietary Ca.	Urinary Ca.	Faecal Ca.	Balance.	Dietary Mg.	Urinary Mg.	Faecal Mg.	Balance.
G. C. N. ...	D I	Nil. 100 mg. manganese as chloride. (MnCl ₂).	P II Average of P III and IV.	203.8	9.8	211.2	-17.2	312.4	37.2	105.8	+169.4
				203.8	26.7	247.6	-70.5	312.4	36.6	108.0	+167.8
P. C. D. ...	D II	Nil. 200 mg. manganese as chloride. (MnCl ₂).	P II Average of P III and IV.	341.5	28.2	288.0	+25.3	341.2	43.3	174.1	+123.8
				341.5	33.4	302.2	+5.9	341.2	60.9	189.2	+91.1

TABLE VI—*concl'd.*

Experimental subject.	Diet.	Supplement.	Period.	PHOSPHORUS METABOLISM.				IRON METABOLISM.				COPPER METABOLISM.			
				Dietary P.	Urinary P.	Faecal P.	Balance.	Dietary Fe.	Urinary Fe.	Faecal Fe.	Balance.	Dietary Cu.	Urinary Cu.	Faecal Cu.	Balance.
G. C. N.	D I	Nil.	P II	944	341	513	+90	48.45	0.61	40.8	+7.04	3.05	0.37	1.28	+1.40
		100 mg. manganese as chloride. (MnCl ₂).	Average of P III and IV.	944	385	536	+23	48.45	0.57	42.5	+5.38	3.05	0.33	1.68	+1.04
V. C. D.	D II	Nil.	P II	1,125	448	541	+136	46.28	0.45	39.3	+6.53	2.73	0.41	1.56	+0.76
		200 mg. manganese as chloride. (MnCl ₂).	Average of P III and IV.	1,125	470	606	+49	46.28	0.41	40.7	+5.17	2.73	0.44	1.78	+0.51

TABLE VII.

Showing the effect of high dose of calcium as lactate to sago diet composed of 550 g. sago, 200 g. sugar and 50 g. ghee (butter-fat) and having a low content of essential minerals.

(The figures indicate the daily average values in mg.)

Experimental subject.	Diet.	Supplement.	Period.	MAGNESIUM METABOLISM.				IRON METABOLISM.			
				Dietary Mg.	Urinary Mg.	Faecal Mg.	Balance.	Dietary Fe.	Urinary Fe.	Faecal Fe.	Balance.
G. C. N. ...	Sago diet {	Nil. 500 mg. Ca as lactate.	P II Average of P III and IV.	43.8	22.2	21.9	— 0.3
				43.8	37.5	115.6	— 109.3
G. C. N. ...	Sago diet {	Nil. 1,000 mg. Ca as lactate.	P II Average of P III and IV.	49.9	21.4	31.7	— 3.2	10.28	0.39	8.78	+ 1.11
				49.9	55.9	233.3	— 230.3	10.28	0.40	18.24	— 8.34
G. C. D. ...	Sago diet {	Nil. 1,000 mg. Ca as lactate.	P II Average of P III and IV.	51.0	17.50	31.8	+ 1.7	9.86	0.29	10.48	— 0.91
				51.0	45.9	142.2	— 137.1	9.86	0.26	16.19	— 6.59
G. C. D. ...	Sago diet {	Nil. 1,000 mg. Ca as lactate.	P II Average of P III and IV.	11.46	0.34	8.42	+ 2.70
				11.46	0.44	18.73	— 7.71

TABLE VII—*concl'd.*

Experimental subject.	Diet.	Supplement.	Period.	COPPER METABOLISM.				MANGANESE METABOLISM.		
				Dietary Cu.	Urinary Cu.	Faecal Cu.	Balance.	Dietary Mn.	Faecal Mn.	Balance.
G. C. N. ...	Sago diet {	Nil.	P II	3.37	0.35	2.08	+ 0.94	0.703	2.006	- 1.393
		500 mg. Ca as lactate.	Average of P III and IV.	3.37	0.34	2.29	+ 0.74	0.703	2.187	- 1.484
G. C. N. ...	Sago diet {	Nil.	P II	2.91	0.20	2.14	+ 0.57	0.735	1.156	- 0.421
		1,000 mg. Ca as lactate.	Average of P III and IV.	2.91	0.31	2.34	+ 0.28	0.735	1.256	- 0.521
G. C. D. ...	Sago diet {	Nil.	P II	2.41	0.54	1.11	+ 0.76	0.694	1.383	- 0.689
		1,000 mg. Ca as lactate.	Average of P III and IV.	2.41	0.61	1.31	+ 0.49	0.694	1.395	- 0.701
G. C. D. ...	Sago diet {	Nil.	P II	2.43	0.45	1.27	+ 0.71	0.714	2.414	- 1.700
		1,000 mg. Ca as lactate.	Average of P III and IV.	2.43	0.49	1.41	+ 0.53	0.714	2.302	- 1.678

Since the elimination through faeces in case of calcium, magnesium, phosphorus, iron and other elements represents not only the unabsorbed food residue but also a large part of utilized minerals which, instead of being eliminated through the kidney, find their way in the intestine, the possibility of the removal of the different elements from the bone, soft tissue or body-fluids due to ingestion of any one of them in excess with the rice and wheat diets having a fair amount of minerals, cannot be ruled out. The negative balances of the different elements which were produced in some cases when any one of them was given in excess with the above diets, support further the possibility of the removal of the minerals from the body proper. This depletion of the body-reserves under the above conditions may not, however, be very large in comparison with the removal of the dietary constituents before absorption through the intestine.

SUMMARY.

1. Investigation carried out by ingesting large doses of different nutritionally essential elements with almost mineral-free sago diet and with rice and wheat diets of normal mineral content has shown that there is mutual antagonistic effect of calcium, magnesium, phosphorus, iron, copper and manganese in metabolism. Administration of a large dose of any of the above elements with the diet increased the elimination of the others to a considerable extent. The increased excretions were found to occur mainly through faeces.
2. Depletion of calcium from the bone after administration of excess magnesium salt and vice versa is probably due to mutual antagonism of these two elements in metabolism.
3. The increased elimination of the different elements found by administering excess calcium salt with the sago diet containing very negligible amount of minerals, is ascribed as due to loss from the body-reserves.
4. In case of rice and wheat diets, containing a fair amount of minerals, the increased elimination of the different elements found by administering one of them in excess with others seems to occur mainly as a result of inhibition of the absorption of the dietary constituents through the intestine.

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ALKALINE PHOSPHATASE IN ERYTHROCYTES.

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THE existence of an alkaline phosphatase in erythrocytes of a few species of mammals has been reported by the authors in a previous publication (Patwardhan and Ranganathan, 1947). Further studies on the enzyme form the subject of the present communication.

EXPERIMENTAL.

Separation of the acid and alkaline phosphatases of the erythrocytes.

Freshly drawn oxalated blood was centrifuged and the plasma removed. The cell layer was stirred up with 7 c.c. to 8 c.c. of 0.9 per cent NaCl solution and centrifuged again. The supernatant saline layer together with as much of the upper buff layer as possible was pipetted out. The washing with saline and removal of the buff layer was repeated four to six times, at the end of which no white blood cell layer was visible. The cells were packed by further centrifuging and the packed-cell volume read off. The cells were then hæmolyzed with the addition of alcohol-water (6 : 4) mixture made up to ten times their packed volume and kept overnight at room temperature. The hæmolyzate was treated with 30 per cent of its volume of chloroform and the mixture shaken vigorously to bring about complete precipitation of hæmoglobin. The alcohol-water layer was removed by centrifuging, followed by filtration. A water-clear solution was obtained in which was found the enzyme. The activity of the preparation (referred to

as preparation A) was tested against sodium glycerophosphate as described below :—

The reaction mixture was made up of 5 c.c. of sodium glycerophosphate (0.88 per cent) + 5 c.c. veronal-sodium acetate-HCl buffer + 0.5 c.c. MgSO_4 0.1 Molar solution + 2 c.c. enzyme preparation. The mixture was incubated at 37°C. for 24 hours, de-proteinized with 25 per cent trichloroacetic acid and the inorganic P determined in the filtrate by the Bell and Doisy (1920) modification of Brigg's method. The results are given in Table I :—

TABLE I.
The phosphatase activity of preparation A.

Source.	Packed volume of r.b.c. in c.c.	Final volume of aq. alcohol layer in c.c.	PHOSPHATASE ACTIVITY IN MG. INORGANIC P IN 24 HOURS.		
			At pH 9*.	At pH 5*.	Blank.
Sheep	1.5	19.5	119	11	4.4
Rat	0.9	12.5	146	12	12
Pig	2.0	28.5	101	4.8	4.8
Rabbit	1.3	18.2	37	...	3.7
Guinea-pig ...	0.75	6.0	143	8	8

* Figures in columns 4 and 5 are corrected for blank.

The method of separation described above resulted in a variable loss of enzyme activity of the alkaline phosphatase together with nearly 90 per cent destruction of the acid phosphatase of the erythrocytes. These effects are described in the following experiment and illustrated in Table II.

The hæmolysate prepared as described above was divided into two halves. One portion was used as such for hydrolysis of sodium glycerophosphate and the other was treated with chloroform as in the previous experiment, the alcohol-water layer being used for hydrolysis. Reaction mixtures were made and treated as already described.

It will be observed that the process of separation met with varying degrees of success. In some cases, there was an inactivation of the alkaline phosphatase which it was not possible to control, the recovery varying from 0 to 100 per cent. The former was particularly the case with dog-blood. Blood from six different dogs has been treated for separation of the enzyme. In every case, there was appreciable activity in the hæmolysate, but after treatment with alcohol-water-chloroform, the resultant solution showed no activity at all.

TABLE II.

The separation of erythrocyte acid and alkaline phosphatases.

Erythrocyte from	HÆMOLYSATE.		PREPARATION A.		PERCENTAGE RECOVERY IN PREPARATION A OF :	
	At pH 9.	At pH 5.	At pH 9.	At pH 5.	Alkaline phosphatase.	Acid phosphatase.
	Mg. inorganic P in 24 hours per 100 c.c. r.b.c.					
Sheep ...	80	211	20.	Nil.	25	Nil.
	77	211	22	Nil.	29	Nil.
	115	211	114	7	99	3
Rat ...	249	222	135	Nil.	54	Nil.
Pig ...	108	136	96	Nil.	89	Nil.
Rabbit ...	40	219	33	33	83	15
Human ...	55	382	35	37	64	10
	113	601	51	67	45	11
Dog ...	75	328	Nil.	Nil.	Nil.	Nil.
	64	332	Nil.	Nil.	Nil.	Nil.

As has been observed earlier, the acid phosphatase is almost completely separated from the alkaline phosphatase by this method. Attempts to extract the former from the protein precipitate have not met with success. Other methods of separation of the acid and alkaline phosphatases, such as adsorption by aluminium hydroxide, magnesium oxide, animal charcoal, *Bleicherde*, Fuller's earth, superfiltrol, etc., have not proved successful.

Activation of the enzyme by Mg-ions.—Jenner and Kay (1931) had shown that phosphatases of mammalian tissues including the erythrocyte phosphatase were activable by Mg-ions. This observation has been confirmed with preparation A from the blood of animals of different species. The activating effect of Mg^{++} was, however, not very marked. The comparative figures for sheep, rat and guinea-pig are given below :—

Preparation A from r.b.c. of :			Without Mg	With Mg
Sheep	11	17
Guinea-pig	12	31
Rat	13	20

EFFECT OF pH ON THE ACTIVITY OF THE ERYTHROCYTE ALKALINE PHOSPHATASE.

The enzyme preparations obtained from sheep and guinea-pig erythrocytes were used for this experiment. Reaction mixtures were made as described before, containing the same buffer of different pH values. The phosphatase activity in terms of inorganic P released at 37°C. in 24 hours is given in Table III :—

TABLE III.

The effect of pH on erythrocyte alkaline phosphatase.

	pH.					
	9.3	8.3	7.4	6.1	5.3	4.2
SHEEP :						
Enzyme preparation A ...	63	29	17	15	15	14
GUINEA-PIG :						
Enzyme preparation A ...	31	23	21	18	17	15
Hæmolysate ...	194	282	582	623	752	942

It will be clear from Table III that in the preparations where the alkaline phosphatase predominates, the highest activity is shown at pH 9.3 within the range tested. The activity decreased as the pH moved towards the acid side; that of the hæmolysate on the other hand was maximum at pH 4.2. Since both the acid and alkaline phosphatases are present in the hæmolysate and the former probably in larger concentration, the activity of the hæmolysate should represent the resultant of the two enzymes acting upon the substrate. The most favourable reaction for the acid phosphatase lies between pH 4 to 5 and hence the highest activity shown by the hæmolysate in that region. The activity of the hæmolysate at pH 9.3 is largely made up of the alkaline component and to a small extent also the residual activity of the acid phosphatase.

EFFECT OF INHIBITORS.

The effect of NaCN, NaF and ZnSO₄ was tested on enzyme preparations from sheep, guinea-pig and rat erythrocytes. The results are given in Table IV. Zinc sulphate gave a small amount of precipitate when added to the reaction mixture at pH 9.3. The experiment was, therefore, repeated with sheep and guinea-pig

r.b.c. preparations at pH 6.1 when also complete inhibition was obtained with ZnSO_4 in M/100 concentration :—

TABLE IV.

The effect of inhibitors on erythrocyte alkaline phosphatase.

Enzyme source.	pH	Inhibitor.	PERCENTAGE INHIBITION AT :		
			M/200.	M/100.	M/50.
Sheep	9.3	ZnSO_4	100	100	100
	6.1	ZnSO_4	86	100	100
	9.3	NaF	24	27	81
	9.3	NaCN	82	88	100
Rat	9.3	NaF	2	2	32
	9.3	NaCN	71	83	89
Guinea-pig	9.3	ZnSO_4	91	100	100
	9.3	NaF	Nil.	Nil.	22
	9.3	NaCN	78	81	95

INFLUENCE OF RICKETS.

Dikshit and Patwardhan (1947) have shown that, in rachitic rats, the serum alkaline phosphatase underwent a marked decrease in activity as compared with the initial values. This observation was contrary to the finding in clinical rickets where the rise in phosphatase activity is diagnostic of even early rachitic process. In view of the fact that an enzyme showing optimum activity in the vicinity of pH 9 existed in the erythrocytes and as the latter are in close contact with the plasma, a study of the erythrocyte and plasma phosphatases as influenced by rickets was considered desirable.

Eighteen rats, four to five weeks old and weighing between 35 g. and 50 g., were kept on the modified Schneider and Steenbock (1939) diet. On the first day, blood was removed by heart puncture in an oxalated tube; the blood from two animals was pooled, the plasma separated by centrifuging and the cell-layer repeatedly washed. It was then haemolysed by dilution with water to ten times the packed-cell volume and allowed to stand overnight in presence of toluene. 0.5 c.c. of plasma was diluted to 10 c.c.

0.1 molar sodium glycerophosphate was used as substrate in presence of 0.005 M MgSO_4 . The hydrolysis was carried out with cellular and plasma enzymes at pH 9 and pH 5 at 37°C. for 24 hours. The results expressed in terms of inorganic P liberated in 24 hours are given in Table V.

The same procedure was repeated at the end of 21 days during which the rats were on the rachitogenic diet. The rats were then sacrificed and the tibial epiphyses examined after silver-nitrate staining.

Six rats from the group were kept as controls. They received from the first day of experiment 320 I.U. of vitamin D per rat per day. Blood was taken from the heart on the first and the twenty-first day, samples from two animals being pooled as before. On the 21st day they were killed and the epiphyses examined.

The results are given in Table V :—

TABLE V.

Effect of rickets on blood phosphatases.

Particulars and rat number.	Degree* of rickets.	ERYTHROCYTE PHOSPHATASE.				PLASMA PHOSPHATASE.			
		ALKALINE.		ACID.		ALKALINE.		ACID.	
		Initial.	Final.	Initial.	Final.	Initial.	Final.	Initial.	Final.
RACHITIC RATS :									
35 and 36 ...	++	139	188	964	1,030	942	518	133	128
37 and 38 ...	++++	113	164	891	916	522	298	136	97
43 and 44 ...	++++	104	110	698	796	617	265	136	112
45 and 46 ...	++++	124	98	769	624	746	275	160	110
47 and 48 ...	++++	83	126	654	772	970	341	152	148
49 and 50 ...	++++	142	105	863	711	637	239	109	105
CONTROL RATS :									
1 and 2 ...	Nil.	203	174	1,121	1,150	578	583	148	117
3 and 4 ...	Nil.	174	137	1,177	1,077	556	535	156	127
5 and 6 ...	Nil.*	...	677	592

1. Activity expressed in terms of mg. inorganic P liberated by 100 c.c. r.b.c. or plasma in 24 hours at 37°C.

2. Control rats received 320 I.U. vitamin D per rat per day.

*Indicated by the width of the epiphyseal cartilage.

DISCUSSION.

The earlier workers Roche and Bullinger (1939), Jenner and Kay (*loc. cit.*) and Behrendt (1943) had tested for the phosphatase activity in the laked r.b.c. The proof of the existence of a separate alkaline phosphatase was not possible under these circumstances as the hæmolysate contained both the acid and the alkaline components. From the data given in Table II it is clear that the procedure adopted in this investigation has resulted in an almost complete separation of the alkaline phosphatase from its acid counterpart. Tables II and III also indicate the fact that the acid phosphatase is far more active than the alkaline component and the optimum pH for the two enzymes are far apart. It is true, however, that in the process of separation, the acid phosphatase undergoes almost complete destruction. The enzyme prepared by alcohol-chloroform treatment has the optimum pH in the alkaline range, is activable by Mg-ions and inhibited by CN^- and Zn^{++} almost completely.

In Table V are given the results of the experiments in which the effect of induced rickets on the cell and plasma phosphatases was investigated. The figures show that in severe rickets the plasma phosphatase showed 43 to 65 per cent decrease in activity over the initial values. As against this, the control animals showed a negligible loss of activity, if any. There were no other changes in the activity of the two cell phosphatases and plasma acid phosphatase attributable to rickets. Dikshit and Patwardhan (*loc. cit.*) had observed that in rachitic rats there was a considerable fall in the activity of the plasma alkaline phosphatase of the rats. This observation has been confirmed in the present investigation. Based on their findings, Dikshit and Patwardhan (*loc. cit.*) had expressed doubts regarding the osseous origin of plasma alkaline phosphatase. As the plasma is in close contact with the red blood cells, it was expected that some correlation between the plasma and erythrocyte alkaline phosphatases would be found. These hopes have not been fulfilled as can be seen from the figures given in Table V. The question of the origin of plasma alkaline phosphatase, therefore, still remains open.

SUMMARY.

1. The presence of an alkaline phosphatase in the red blood cells of sheep, rat, pig, guinea-pig and man has been demonstrated.
2. The alkaline component of the erythrocyte phosphatases has been separated from the acid phosphatase and its conditions of action studied.
3. Zinc sulphate and sodium cyanide have been shown to exert a powerful inhibiting action on the phosphatase in M/200 concentrations. Sodium fluoride was found to be comparatively a weak inhibitor.
4. In rat rickets, the plasma alkaline phosphatase shows a very large decrease in activity whereas the erythrocyte alkaline phosphatase shows no appreciable change.

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STUDIES ON SNAKE VENOMS.

Part I.

THE ISOLATION OF AN INHIBITOR OF THE CYTOCHROME- CYTOCHROME OXIDASE SYSTEM FROM COBRA VENOM AND STUDY OF THE EFFECTS OF pH, TEMPERA- TURE AND ULTRA-VIOLET RAYS ON ITS STABILITY.

BY

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It has been reported by Ghosh and Chatterjee (1948) that cobra venom contains an active principle responsible for the inhibition of the cytochrome-cytochrome oxidase system in tissue-cells and that the active principle can be partially separated from the other constituents associated with it in the venom. The present communication deals with the attempt at isolation of the active principle from the crude venom and a study of its chemical properties.

EXPERIMENTAL.

Venom used.—The venom used was that of the cobra. The toxicity of the venom, as determined by intramuscular injection into pigeons weighing about 300 g., was 0.1 mg.

Measurement of the activity of the inhibitor of cytochrome oxidase system.—The activity of the inhibitor of the cytochrome oxidase system at the different stages of its isolation from the venom was measured by comparing its effect on the cytochrome oxidase system with that produced by the venom. As a source of the enzyme system, pigeon's brain tissue was employed. Immediately after killing

the animal, its brain was taken out, cooled on ice for several minutes and then chopped and minced to a fine paste.

The technique of experiment.—Approximately 50 mg. portions of the finely minced and chopped pigeon's brain were accurately weighed out and placed in separate Warburg's flasks containing phosphate-Ringer-Locke solution of pH 7.4 prepared by the method of Passmore *et al.* (1933). An adequate amount of the venom or of the inhibitor also dissolved in phosphate-Ringer was added to the brain suspension. Suitable controls were kept. 1.0 c.c. of 0.5 per cent solution of p-phenylene diamine hydrochloride in phosphate-Ringer, adjusted to pH 7.0, was kept in the side tubes where necessary. 0.2 c.c. of 20 per cent KOH solution was kept in the inner cup of each flask together with a roll of filter-paper. The total volume of liquid in each flask was 3.0 c.c. The flasks were fitted to the manometers and shaken in a thermostat at $37.5^{\circ} \pm 0.1^{\circ}\text{C}$. for 1 hour and 30 minutes in presence of air. By this process, the brain was depleted of the major part of the substrates originally present in it and, moreover, sufficient time was given to the venom to produce its full effect. After the period of incubation was over, the substrate solution was tipped off from the side tube into the main compartment, the level of liquid in the manometers was adjusted, the taps closed and the oxygen consumption was determined for 1 hour at intervals of 15 minutes. From the figures for oxygen consumption, the degree of inhibition of cytochrome oxidase system by the venom or the inhibitor was calculated.

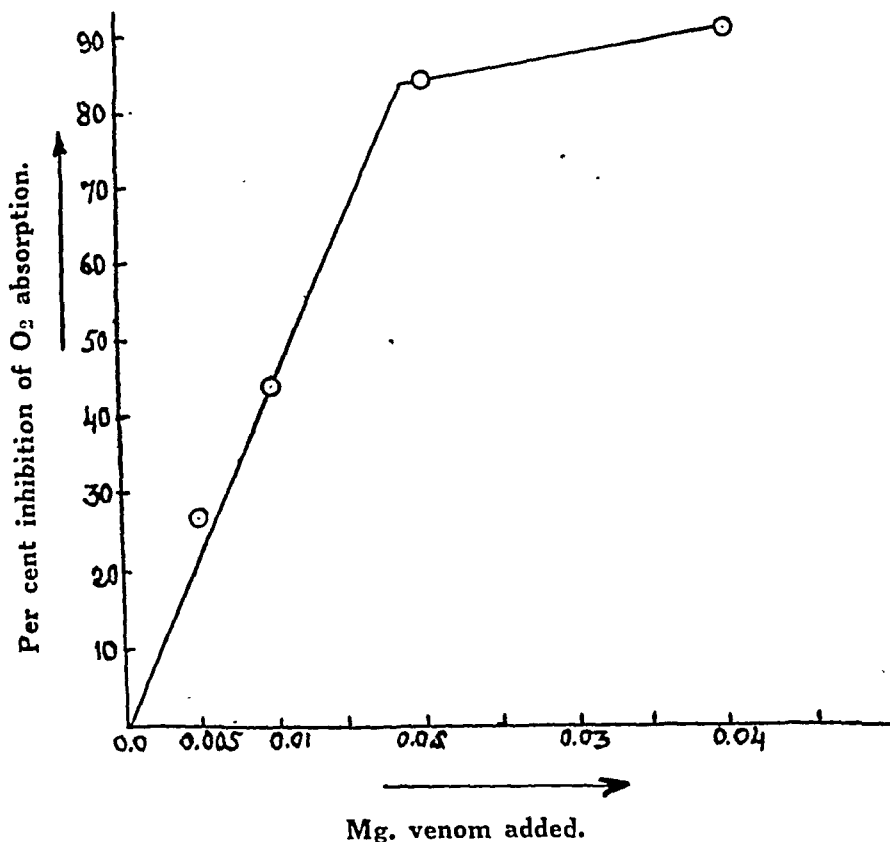
Choice of a suitable unit.—For comparing the effect of the venom on the cytochrome oxidase system with that of the inhibitor, a suitable dose of the venom had to be chosen as the unit. In order to determine this unit, the effects of varying doses of the venom on cytochrome oxidase system were investigated by the technique described above. Table I shows the degree of inhibition of oxidation of p-phenylene diamine by pigeon's brain in presence of different doses of cobra venom:—

TABLE I.

The effect of varying doses of the venom on cytochrome oxidase.

Number.	Amount of venom used, mg.	Per cent inhibition of the oxidation of p-phenylene diamine.
1	0.005	27.0
2	0.010	44.0
3	0.020	84.0
4	0.040	90.0

GRAPH.



Inhibition of the action of cytochrome oxidase by different doses of cobra venom.

The relationship between the amount of venom used and the degree of inhibition produced is shown in the curve. It is evident that up to 0.02 mg. of venom, the percentage of inhibition is linearly related to the amount of venom. Above 0.02 mg., however, the curve becomes flattened. Thus, any dose of venom up to 0.02 mg. can be taken as the unit. For convenience, the unit was defined as that amount of venom or of the inhibitor, which when allowed to act on 50 mg. of finely chopped and minced pigeon's brain-tissue for 1 hour and 30 minutes at 37.5°C . in air, would subsequently inhibit the oxidation of 0.166 per cent solution of p-phenylene diamine hydrochloride (neutralized) by the brain to the extent of 45 per cent to 50 per cent in 1 hour. By trial, the unit was found to be 0.01 mg. of dry cobra venom.

Separation of the active principle.—One gramme of crude cobra venom, dissolved in 100 c.c. of distilled water, was adjusted to pH 9.0 and treated with

20 g. of pure NaCl. The solution was kept warm for about 30 minutes and then filtered. The precipitate was dissolved in 50 c.c. of water, the pH adjusted to 9.0 and 10 g. of NaCl were added. The solution was filtered off and this filtrate combined with the first one. To the combined filtrate, dilute H_2SO_4 was added to lower the pH to 2.0 and the solution was filtered off. The precipitate was dissolved in 50 c.c. of water, the pH adjusted to 3.8 and 8 g. of anhydrous Na_2SO_4 were added in small amounts at a time. The solution was filtered and the filtrate was set aside. The precipitate was dissolved in 25 c.c. of water at pH 3.8 and 4 g. of anhydrous Na_2SO_4 were slowly added. It was filtered and the precipitate was discarded. The combined filtrates were treated with 4.5 g. of anhydrous Na_2SO_4 and filtered. The solution was filtered and the filtrate rejected. The precipitate was dissolved in 10 c.c. of water, the pH adjusted to 6.0, the solution cooled to 1°C . and treated with 15 c.c. of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$. The solution was filtered and the filtrate rejected. The residue was dissolved in 10 c.c. of water and the solution was cooled to 1°C . and the pH adjusted to 4.0 and 8.5 c.c. of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ were added drop by drop. After allowing the precipitate to settle for about an hour at 1°C . it was filtered off and dried. This final precipitate had an activity of 33 per cent and a protein content of 2.12 per cent with respect to crude venom. Thus, for the same nitrogen content it was 16 times more active than the crude venom. It was found to be completely free from hæmolysin and choline esterase, but was contaminated with traces of cardio-toxin.

Table II shows the relative percentages of protein and the inhibitor at different stages of its isolation from the crude venom:—

TABLE II.

Stage.	Precipitate and how it was obtained.	PROTEIN.	ACTIVITY.
		Per cent.	Per cent.
1	Crude venom	91.70	100
2	Precipitate obtained from the venom solution at pH 2.0, after removing some of the proteins by 20 per cent NaCl at pH 9.0.	45.60	90
3	Precipitate obtained from the solution of the precipitate (2) by 22 per cent Na_2SO_4 at pH 3.8, after rejecting the precipitate with 16 per cent Na_2SO_4 at the same pH as above.	7.52	55
4	Precipitate obtained by dissolving the precipitate (2) and precipitating with 22 per cent Na_2SO_4 at pH 3.8.	5.95	50
5	Precipitate obtained by dissolving the precipitate (4) in 10 c.c. of water and precipitating with 15 c.c. of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ at pH 6.0.	3.48	35
6	Precipitate obtained by dissolving the precipitate (5) in 10 c.c. of water and precipitating with 8.5 c.c. of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ at pH 4.0.	2.12	33

Preparation of the salt-free inhibitor.—The inhibitor separated from crude cobra venom was contaminated with inorganic salts like NaCl, Na_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$, etc. To remove these, the process of dialysis was taken recourse to. The inhibitor was dissolved in ice-cold water and dialysed through a cellophane sac against cold water inside a frigidaire. The water was replaced at frequent intervals to facilitate the process of dialysis. When the dialysate showed no test for chloride and sulphate ion, the sac was emptied and the protein solution was dried in vacuum inside a frigidaire.

The toxicity of the salt-free inhibitor.—The m.l.d. of the inhibitor as determined by intramuscular injection into pigeons weighing between 300 g. and 310 g. was found to be 0.3 mg.

Influence of temperature on the activity of cobra venom and of the inhibitor.—Of the different factors that affect the activity of proteins, the effect of temperature is none the less important. The effects of temperature on the activity of cobra venom and of the inhibitor were investigated with a view to throwing some light on their chemical nature. For the purpose, 0.1 and 0.06 per cent solutions of cobra venom and of the inhibitor respectively in phosphate-Ringer pH 7.4, were heated at various temperatures for half an hour. The solutions were then cooled, suitably diluted and the activity determined by the method already described. The results are shown in Tables III and IV :—

TABLE III.

The effect of temperature of the activity of cobra venom.

Number.	Period of exposure, minutes.	Temperature of exposure, °C.	Per cent inactivation.
1	30	70	0
2	30	75	0
3	30	80	10
4	30	83	55
5	30	85	100

TABLE IV.

The effect of temperature on the activity of the purified inhibitor.

Number.	Period of exposure, minutes.	Temperature of exposure, °C.	Per cent inactivation.
1	30	70	0
2	30	75	0
3	30	80	56
4	30	83	100

It will be observed from the results in Tables III and IV that the purer the inhibitor the more rapidly it is destroyed by heat. The half inactivation temperature of the impure inhibitor, i.e. the crude venom, is about 83°C., whilst that of the purified inhibitor is about 80°C. These observations are in accord with that of Willstatter, Grassner and Kuhn (1902) that the effect of temperature on an enzyme is largely dependent on its purity. In the case of crude venom, the proteins accompanying the inhibitor probably exert a protective effect upon it.

Irreversible nature of inactivation by heat.—In order to determine whether the inactivation of the inhibitor by heat is reversible or not, the partly inactivated solutions of these substances in phosphate-Ringer, pH 7.4, were kept in the frigidaire at 4°C. for 48 hours and their activities were determined by the usual method. It was found that the partly inactivated solutions did not show any increase of activity, proving thereby that the inactivation was completely irreversible in nature.

Influence of H-ion concentration on the activity of the purified inhibitor.—Like temperature, the pH of the medium has a profound influence on the activities of various toxins. The stability of the inhibitor of cytochrome oxidase at different pH values of the medium was, therefore, investigated. 0.06 per cent solutions of the inhibitor were adjusted at different pH values and kept for different periods of time in the frigidaire. In order to adjust the pH to 1.0, a definite volume of the inhibitor solution was mixed with an equal volume of N/5 HCl. Similarly, for pH 13.0 equal volumes of the solutions of the inhibitor and N/5 NaOH were mixed together. After exposing the inhibitor to different H-ion concentrations for known periods of time, the solutions were neutralized, diluted and the activity determined. The results are shown in Table V:—

TABLE V.

The effect of pH upon the activity of the inhibitor of cytochrome oxidase.

Number.	pH at which exposed.	ACTIVITY IN PER CENT OF THE ORIGINAL AFTER EXPOSING IT FOR :		
		1 day.	2 days.	3 days.
1	1.0	96	82	78
2	3.0	100	96	90
3	5.0	100	98	94
4	6.0	100	98	98
5	7.4	100	100	100
6	9.6	98	95	88
7	13.0	0

From the results recorded in Table V it will be noticed that the inhibitor isolated from cobra venom is fairly stable at different pH values, the stability being maximum at pH 7.4. The stability is reduced at pH above and below 7.4, becoming very low at pH 13.0, at which it is inactivated in the course of a single day.

Irreversibility of inactivation.—To determine the nature of pH inactivation the inactivated solutions were brought to pH 7.4 and kept in the frigidaire at 4°C. for 48 hours and then the activity of the solutions was tested. No gain of activity was noticed and hence it was concluded that the inactivation was completely irreversible.

Effect of ultra-violet rays on the stability of the inhibitor.—Of the various agents affecting the properties of proteins, radiation of short wave-lengths occupies an important position. The different active principles of cobra venom, e.g. hæmolysin, choline esterase, and cardio-toxin are all known to lose their activities when exposed to ultra-violet radiation. The effect of ultra-violet rays on the activity of the inhibitor of cytochrome oxidase was, therefore, investigated.

A 0.06 per cent solution of the salt-free inhibitor in phosphate-Ringer, pH 7.4 was kept at a distance of about 30 cm. from a mercury vapour lamp for a period ranging from 15 minutes to 2 hours. Another solution of the same concentration was kept unexposed to serve as control. After the exposure was complete, the solution was diluted suitably and the activity compared with that of the control. The results are shown in Table VI:—

TABLE VI.

The effect of ultra-violet rays on the activity of the inhibitor.

Number.	Period of exposure, minutes.	Activity in per cent of the original after exposure.
1	15	100
2	30	100
3	60	100
4	120	102

The results indicate that the ultra-violet rays have no effect on the inhibitor of cytochrome oxidase isolated from cobra venom.

SUMMARY.

1. An active principle responsible for the inhibition of the cytochrome oxidase system has been separated from cobra venom. Weight for weight, the purified product is 16 times more active than the crude venom.

2. The effects of certain inactivating agents like temperature, pH and ultra-violet rays on the stability of the inhibitor of the cytochrome oxidase system have been studied. The inhibitor is fairly stable at moderately high temperatures and is unaffected by ultra-violet rays even when the exposure lasts for 2 hours. It is also quite stable within the range of pH 1.0 to pH 9.6.

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TREATMENT OF TYPHOID FEVER WITH BACTERIOPHAGE.

A PRELIMINARY REPORT.

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INTRODUCTION.

ENTERIC fever has been endemic in the city of Bombay for a number of years. Occasionally it flares up in an epidemic form all over the city as it did in the year 1938, causing 1,168 reported attacks during the year with 507 deaths—a fatality rate of 43·4 per cent. Table I giving the incidence of enteric fever in Bombay during a recent five-year period (Bombay Municipal Health Records, 1942-46) shows that though its spread has been in check, the number of attacks and the fatality rate show no signs of abatement :—

TABLE I.
Incidence of enteric fever in Bombay.

Year.	Reported attacks.	Deaths.	Fatality rate, per cent.	Death rate per 1,000 of population.
1942 ...	1,018	375	36·8	0·2
1943 ...	1,562	567	36·6	0·4
1944 ...	1,008	459	45·5	0·3
1945 ...	1,329	616	46·3	0·4
1946 ...	988	513	51·9	0·3

The figures quoted under-estimate the true state, as quite a number of cases is not reported to the authorities or not recognized. A great majority of the enteric fever cases in Bombay is of typhoid fever. Only about 10 per cent of the cases are caused by *S. paratyphi* A. Enteric fevers caused by *S. paratyphi* B or *S. paratyphi* C are practically non-existent in Bombay (unpublished data). The majority of the cases occurs during the monsoon season (i.e. during the months from June to October), but quite an appreciable number of cases is also met with during the other months.

In 1940, when a typhoid epidemic was raging in the North of Bombay Island, an investigation was started by one of us (R. G. D.) with a view to find out whether the proximity of the Dadar Sewage Purification Works was in any way responsible for it. As a result of that work, it was shown that *S. typhi* was almost constantly present in the sewage and in the effluent which flowed out of it after purification. From the sewage, later, was also isolated a bacteriophage which was found to act specifically against *S. typhi* in high dilutions (Dhayagude, 1943).

Ever since the discovery of the phenomenon of bacteriophagy by Twort and d'Herelle, it was believed that a potent therapeutic weapon had become available against certain bacterial diseases. It was only a question of finding out an appropriate 'phage of adequate strength. This feeling was entertained not only among the practitioners but also among the scientists due to the fact that the phenomenon of bacteriophagy as observed *in vitro* was such a spectacular one. Numerous workers have tried bacteriophage therapy in typhoid fever with varying results, and its claims are yet neither proved nor disproved. It was, therefore, decided to test the therapeutic effect of the locally isolated 'phage on typhoid-fever patients in Bombay. In the earlier part of the investigation the 'phage was administered orally, while later it was administered intravenously.

Asheshov, Wilson and Topley (1937) reported on the protective effect in mice of typhoid 'phage given separately and by routes different from those used in infecting the animals. They found that the specific anti-Vi 'phage was a very effective protective agent, while the non-specific 'phage had no protective effect. Fisk (1938) not only confirmed this protective action but also showed that the 'phage had a distinct therapeutic or curative effect. Ward (1943) confirmed Fisk's results and pointed out the apparent multiplication of the 'phage *in vivo* in mice.

On the strength of the above experimental data, the treatment with type-specific Vi typhoid bacteriophage administered intravenously was tried at the Los Angeles County General Hospital as reported by Knouf *et al.* (1946) with highly beneficial results. In India, Banerjee (1939) treated three cases of typhoid by administering bacteriophage intravenously. Though a type-specific Vi 'phage was not used, the results were 'astonishing'. When the present study was undertaken, we did not have facilities to determine whether the typhoid 'phage we had was an 'O' 'phage or a Vi 'phage. Experiments, however, showed that our stock 'phage was quite potent in lysing the cultures of *S. typhi* isolated from many different sources and it was, therefore, decided to try the effect of its intravenous administration on a few patients, using the same technique as that described by

Knouf *et al.* (*loc. cit.*). The stock 'phage was derived from the original 'phage isolated by one of us (R. G. D.) from the local sewage. This was maintained in proper strength by propagating it at regular intervals on a stock strain of *S. typhi* isolated from the sewage simultaneously with the 'phage. In a broth tube the 'phage caused apparently complete lysis of a young culture within 3 hours but after a further incubation of 24 hours a slight growth of resistant forms of the organisms could be seen. The number of 'phage particles per millilitre as determined on an agar plate was found to be 10^{12} .

The problem was taken up for research under the auspices of the Indian Research Fund Association from the 1st of October, 1946. Before this, 30 cases of typhoid had already been treated with bacteriophage by mouth, the results of which have been reviewed elsewhere (Banker, 1946).

SELECTION OF CASES.

The patients selected for treatment were from among those admitted at the City Fever Hospital, and from those admitted to a special Enteric Ward at the King Edward VII Memorial Hospital, Bombay. The former group of patients were all given the 'phage by the oral route, while the patients at the second hospital were given the 'phage intravenously as advocated by Knouf *et al.* (*loc. cit.*). This was because adequate nursing facilities and constant personal supervision were possible at the latter hospital.

The following criteria were strictly observed in the selection of the cases taken up for oral therapy:—

1. The cases admitted for fever were first clinically examined, and if suspected to be typhoid, the blood was collected for culture. The diagnosis in all cases was made by means of a positive blood- or clot-culture for *S. typhi*.
2. Only cases admitted during the first week of their fever were taken up for the cultural examination of the blood, so that the treatment which in these cases lasted a number of days could be instituted early.
3. The 'phage was in every case tested against the isolated strain and was administered only if that culture was lysed.
4. As the oral treatment had to extend several days to be effective, if at all, it was seen at the time of starting the treatment that (a) the patient had no serious complications and (b) that he had not received any other special treatment, such as penicillin or sulphathiazole.
5. The bacteriophage treatment was abandoned if the patient developed any serious complications during the treatment which might necessitate administration of other drugs, such as penicillin.
6. Every alternate case diagnosed positive by blood-culture was kept as a 'control' and administered plain 'broth' in which the bacteriophage was prepared.

7. The cases were taken up irrespective of their age, sex, build or previous history of inoculation. Treating every alternate case strictly as a control was expected to eliminate such factors as nutrition, age, sex, etc., interfering with the true assessment of results.

Similarly, the following criteria were observed in the selection of the cases taken up for *intravenous* therapy :—

1. The diagnosis, as in the *oral* series, was made only when *S. typhi* could be isolated from the culture of blood or clot of suspected patients.
2. The patients were taken up for treatment whatever the duration of the fever, provided the blood-culture was positive and the strain of *S. typhi* isolated was lysed by the stock 'phage.
3. Since the *intravenous* therapy lasted only one day, and the beneficial results, if any, were expected to be prompt, the treatment was started despite any complication that might be present, or despite any other treatment the patient might have had, provided the patient was not in a moribund state.
4. One-third of the positive-diagnosed cases were taken up for treatment. Every third case was kept as a 'control'. The control cases were not given any special treatment besides the general nursing, except that in the last control case 'autolysate' was given *intravenously*. Autolysate consisted of filtered products of 24-hour broth culture of *S. typhi* isolated from the blood of the patient to whom it was to be given.
5. The remaining third of the positive-diagnosed cases were given sulphathiazole-penicillin treatment, and formed part of a separate investigation (to be published).
6. As in the *oral* series, the patients were taken up for treatment irrespective of their age, sex, nutrition or previous history of inoculation.
7. Some cases, despite positive culture, were clinically found to be very mild and these were not taken up for treatment.
8. In no case was combined *oral* and *intravenous* 'phage treatment given.
9. The blood-culture was repeated in each case treated by *intravenous* bacteriophage 48 hours after the treatment was concluded.

A BRIEF ANALYSIS OF THE CASES.

During the course of the investigation, 184 suspected cases were taken up for cultural examination of the blood. *S. typhi* was isolated from 79 of these patients, while *S. paratyphi* A was isolated from 10 patients. Of the 79 typhoid patients, the actual number taken up for treatment was 24, 10 being given the 'phage orally

and 14 intravenously. A corresponding number of cases was kept as control, as represented in Table II :—

TABLE II.

Distribution of the cases.

	Total cases.
Blood- or clot-culture positive for <i>S. typhi</i> ...	79
Treated with 'phage orally ...	10
Controls given broth orally ...	10
Treated with 'phage intravenously ...	14
Controls, untreated (except for 1 given autolysate) ...	14
Not included in the present investigation ...	31

The group of 31 cases not included in the present investigation is made up of 16 who were taken up for sulphathiazole-penicillin treatment, and the rest 15 who were not found suitable for applying any therapy, either because they exhibited a very mild course or because they were in a moribund state at the time of admission and died soon after.

The age, sex, and other relevant data regarding the 24 'treated' and the corresponding 24 'control' cases are summarized in Tables III and IV. In the *intravenous* series, besides repeated blood-cultures, the faeces and the urine were also cultured repeatedly, and the patient was not discharged till three consecutive examinations were negative. In the *oral* series, the faeces and the urine were not put up for cultural examination.

TREATMENT WITH BACTERIOPHAGE ADMINISTERED ORALLY.

Each patient, on his blood-culture proving positive for *S. typhi*, was given 50 c.c. 'phage in two equally divided doses, one early in the morning and the other 12 hours later in the evening. Half an hour before the administration of 'phage, 30 grains of sodii bicarbonatis were given every time by mouth with a view to render the stomach contents alkaline. The treatment was continued till the temperature was normal for three consecutive days. In none of the cases did any reaction occur which could be directly ascribed to the bacteriophage. No spectacular results to resemble the *in vitro* lysis were obtained in any case.

Besides this special bacteriophage treatment, all the patients were given a uniform standard nursing treatment, and the complications were treated as they arose.

TREATMENT WITH BACTERIOPHAGE ADMINISTERED INTRAVENOUSLY.

In each case, as soon as the blood-culture was found positive and it was decided to take up the patient for intravenous 'phage therapy, the patient's sensitivity to the protein of the bacteriophage was tested by an intradermal injection of 0.1 c.c. of 1 in 10 diluted 'phage. The 'phage was then administered intravenously the next day, i.e. 24 hours later, if there was no appreciable local reaction. None of the 14 cases showed a reaction on intradermal injection, which might indicate hypersensitiveness.

The dosage and the procedure followed were as advocated by Knouf *et al.* (*loc. cit.*). A single dose of 1 c.c. of the 'phage (1.5 c.c. to 2 c.c. in some of the later cases) was given by intravenous drip diluted in about 500 c.c. of 5 per cent glucose-saline solution, taking about four to five hours to run in completely. The temperature (axillary), pulse and respiration were charted every 15 minutes and the blood-pressure recorded every hour.

The expected reaction, supposed to be due to widespread lysis of the organisms, occurred in one to three hours after the commencement of the therapy. The patient usually got a rigor lasting for about half an hour, the temperature quickly rising to about 105°F. (in the axilla). In some cases the reaction was severe enough to cause momentary anxiety regarding the state of the patient. At this stage, to avoid any chance mishap, vasoconstrictors, such as desoxycorticosterone, were injected intramuscularly, and nikethamide added to the transfusing solution. The patient was wrapped up for a few hours in wet packs to prevent the temperature from rising too high. The temperature almost invariably came down to 97°F. (in the axilla) in 12 to 24 hours after the conclusion of the therapy, and often went down to 95°F. for a few hours. In most cases the temperature, however, failed to persist at the normal level.

Besides this special treatment, all cases were given a uniform standard nursing treatment. Light solid diet, such as bananas and soft bread, were started as soon as the patient felt hungry and able enough to feed himself, which was very often quite early in the course of the illness. This régime, it is believed, helped to shorten the convalescence.

RESULT OF ORAL THERAPY.

The number of cases treated has been very few, yet the result clearly indicates that there was no spectacular effect. Moreover, since strict controls have been kept, an attempt may be made to compare the results in the two groups. It can be seen from Table V that the mortality and the incidence of major complications (as intestinal hæmorrhage, perforation, hyperpyrexia, meningism, relapse, etc.) are actually higher in the 'phage-treated cases than in the controls. The difference in toxicity before and after treatment is indicated in Table III and a comparison with the controls is shown in Table V.

TABLE III.

Oral cases.

Number.	Age.	Sex.	Started, day.	Stopped, day.	Duration of fever, day.	Toxiæmy.		Complications.	Result.
						Before.	During.		
(A) Treated with bacteriophage.									
1	17	F.	9th	34th	31	++	+++	Stomatitis, broncho- pneumonia.	C
2	22	M.	9th	23rd	20	++	+	Nil	C
3	22	F.	8th	18th	15	++++	++	Stomatitis, broncho- pneumonia.	C
4	23	M.	6th	23rd	20	++	Nil	Nil	C
5	30	M.	8th	16th	...	+	+++	Peripheral failure	E
6	9	M.	9th	21st	18	+	Nil	Parotitis	C
7	26	F.	7th	12th	9	Nil	Nil	Diarrhoea	C
8	33	M.	7th	13th	10	Nil	Nil	Nil	C.
9	20	M.	8th	19th	16	Nil	Nil	Nil	C
10	16	M.	8th	11th	...	Nil	+++	Intestinal hæmorrhage	E

TABLE IV—*concd.*

Number.	Age.	Sex.	Day of therapy.	Toxicity.		Blood-culture.		Duration of fever, days.	Complications.	Result.
				Before.	After.	Before.	After.			
(B) 'Controls'—no special treatment (except autolysate to No. 14).										
1	30	M.	...	+	+++	—	—	...	Pneumonia	E
2	30	M.	...	+	+++	—	—	...	Hyperpyrexia	E
3	28	M.	...	+	+	—	—	54	Internal hæmorrhage.	C
4	25	M.	...	+	Nil	—	—	30	Nil	C
5	24	F.	...	+	+++	—	—	49	Diarrhoea, deafness, bed sores.	C
6	16	F.	...	Nil	++	—	—	46	Internal hæmorrhage, hyperpyrexia, bed sores.	C
7	25	M.	...	+	++	—	—	24	Nil	C
8	20	M.	...	+	+++	—	—	29	Internal hæmorrhage.	C
9	28	F.	...	++	Nil	—	—	18	Relapse	C
10	14	M.	...	+	++	—	—	32	Nil	C
11	40	F.	...	+	+	—	—	18	Internal hæmorrhage.	C
12	25	M.	...	+	+++	—	—	...	"	E
13	22	M.	...	+	+++	—	—	62	Deafness, relapse, bed sores.	C
14	30	M.	35th	Nil	Nil	+	+	54	Nil	C

RESULT OF INTRAVENOUS THERAPY.

In 2 of the 14 cases treated there was a fairly remarkable cure, the temperature having come down to normal within 24 hours of the therapy by crisis, and the blood-culture and repeated cultures of the urine, and faeces remaining negative subsequently. It must be added, however, that these 2 cases were not in a particularly serious state when the therapy was started. The only death in the series was of the patient who died a few days after the therapy due to complicating pneumococcal septicaemia. The remaining 11 patients recovered, and though the temperature in some cases showed a lowering of the maximum range, the course did not appear to be shortened. In 4 of these, the blood-culture became negative and the toxicity diminished soon after the therapy, while the urine- and faeces-cultures also remained negative. A comparable diminution in toxicity with negative urine- and faeces-culture was obtained in only one case from the control group (this patient had a relapse subsequently). In 7 cases the subsequent blood-cultures after the therapy remained positive for *S. typhi*, nor was there any appreciable decrease in the general toxicity. Among the control cases which were not given any special treatment, there were 8 which were only mildly toxic at the time of admission but developed marked toxicity after about seven days; three of these expired.

One control case, which was administered autolysate intravenously, developed a rigor very much like the 'phage-treated cases (though the autolysate did not contain any 'phage), and his temperature came down to normal and remained so for 24 hours. His blood-culture, however, remained positive and the temperature subsequently rose to previous levels.

TABLE V.

Result in treated cases compared with controls.

		Total.	Major complications.	Expired.	TOXICITY DURING 7 DAYS.		
					Increased.	Decreased.	Same.
Orally treated	...	10	6	2	3	4	3
Controls (broth)	...	10	1	1	5	2	3
Intravenously treated		14	7	1	1	6	7
Controls	...	14	10	3	8	2	4

Case reports.—The following interesting case reports are presented here in brief:—

1. Mrs. M. M., age 20 years, female, wife of the case quoted below, was admitted on 27th March, 1947 for continuous fever of 8 days' duration, cough and vomiting. On examination, she was found to be slightly toxic. The tongue was coated and the spleen was not palpable. Temperature 102°F. (axillary), pulse 90 and respiration 26 at the time of admission. Blood- and clot-cultures were both positive for *S. typhi*. The bacteriophage was administered by intravenous drip on the 12th day of fever. At the time of starting, the general condition of the patient was fair, she was on liquid feeds, had no complications and was quite co-operative. The blood-pressure was 110/60 mm. Hg., T. 100.6°F. (axillary), P. 112, and R. 28.

At the end of one hour, the patient had a severe rigor lasting for about 10 minutes and she became very anxious. The pulse volume during the rigor became very feeble. The temperature then started rising, the rigor became mild to last 20 minutes more, and the pulse volume became normal. In half an hour the temperature rose to 104°F. in the axilla, and then started falling gradually. It touched 97°F. about 8 hours after the 'phage was started. Except for a short rise 48 hours later, the temperature remained normal throughout, and the patient had an uneventful recovery (see Temperature Chart 1). The very next day after the 'phage therapy she complained of intense hunger and was immediately started on light solid foods such as soft bread, biscuits and bananas. Blood- and clot-cultures taken 24 hours later were both negative. The urine- and faeces-cultures also remained negative throughout the convalescence. She was discharged cured on 25th day of her illness.

2. Mr. A. M., age 25 years, male, husband of the case reported above, was admitted on 4th April, 1947 for continuous fever of 4 days' duration, headache, constipation, epistaxis, pain in the abdomen and joints. He was hospitalized 7 days after the date of admission of his wife. He had been given 1 c.c. of T.A.B. vaccine subcutaneously on 26th March, 1947 on it being suspected that his wife was suffering from typhoid. On examination, he was not found to be toxic, and the spleen was not palpable. T. 101°F. (axillary), P. 90, and R. 20. Blood- and clot-cultures were both positive for *S. typhi*. He was kept as a control and not given any but the basic nursing treatment. The fever in this case continued for 28 days, the temperature coming down to normal on 28th April, 1947. During the course of the illness, about the 14th day of fever, the patient developed a fair degree of toxicity and was delirious. His convalescence was uneventful but prolonged. He was discharged cured on 46th day of his illness.

DISCUSSION.

The stock bacteriophage used in the present investigation was tested recently and found to be an 'O' 'phage and not a 'Vi' 'phage, i.e. it was found active against the 'O' antigen but not specifically against the 'Vi' antigen of *S. typhi*. Though this 'phage was able to lyse all the *S. typhi* cultures isolated from the various patients, it is now felt that admittedly it was not an ideal agent for therapy.

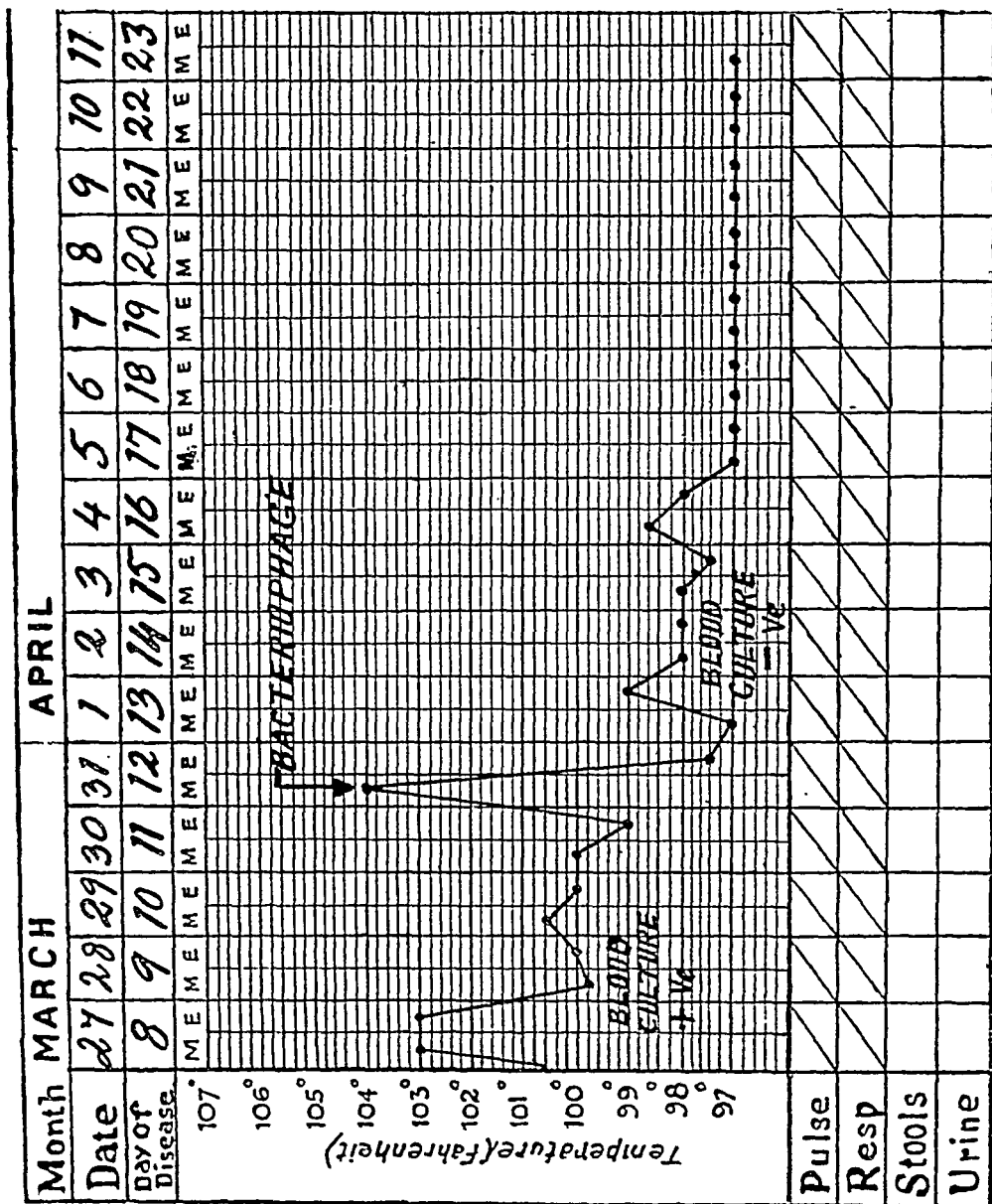
The 'phage used having been a stock 'O' 'phage, the results obtained with the intravenous therapy are not comparable with those reported by Knouf *et al.* (*loc. cit.*), after using type-specific Vi bacteriophage. They, however, compare well with the results obtained by the same workers during a period when they used unselected stock bacteriophage for intravenous therapy (reported by Bower, 1938, quoted by Knouf *et al.*, *loc. cit.*). In their words, 'some (patients) seemed to derive no benefit whatever, while others proceeded with obviously or at least an apparently modified course, and some achieved truly startling bacteriologic and clinical recovery.. Results in the latter patients were so spectacular that they caused us to continue the study despite the discouragement of many failures and indifferent successes, for they seemed to indicate an unknown factor which might, when discovered, lead to more uniformly good results'. The unknown factor, according to these workers, is probably the application of type-specific Vi bacteriophage. In India, Banerjee (*loc. cit.*) successfully treated three cases of typhoid fever by administering the 'phage intravenously. All were bacteriologically proved cases of typhoid, and the 'phage, which was given in one injection and not by a continuous drip method, was repeated on two or three successive days. The 'phage used was a stock unselected 'phage and not a type-specific Vi one.

On the strength of the experience and results with oral administration of the 'phage to 30 cases reported previously (Banker, *loc. cit.*) and to 10 cases in the present series, with as many cases kept strictly as controls, it may be stated that oral therapy with an unselected stock bacteriophage such as we used has no

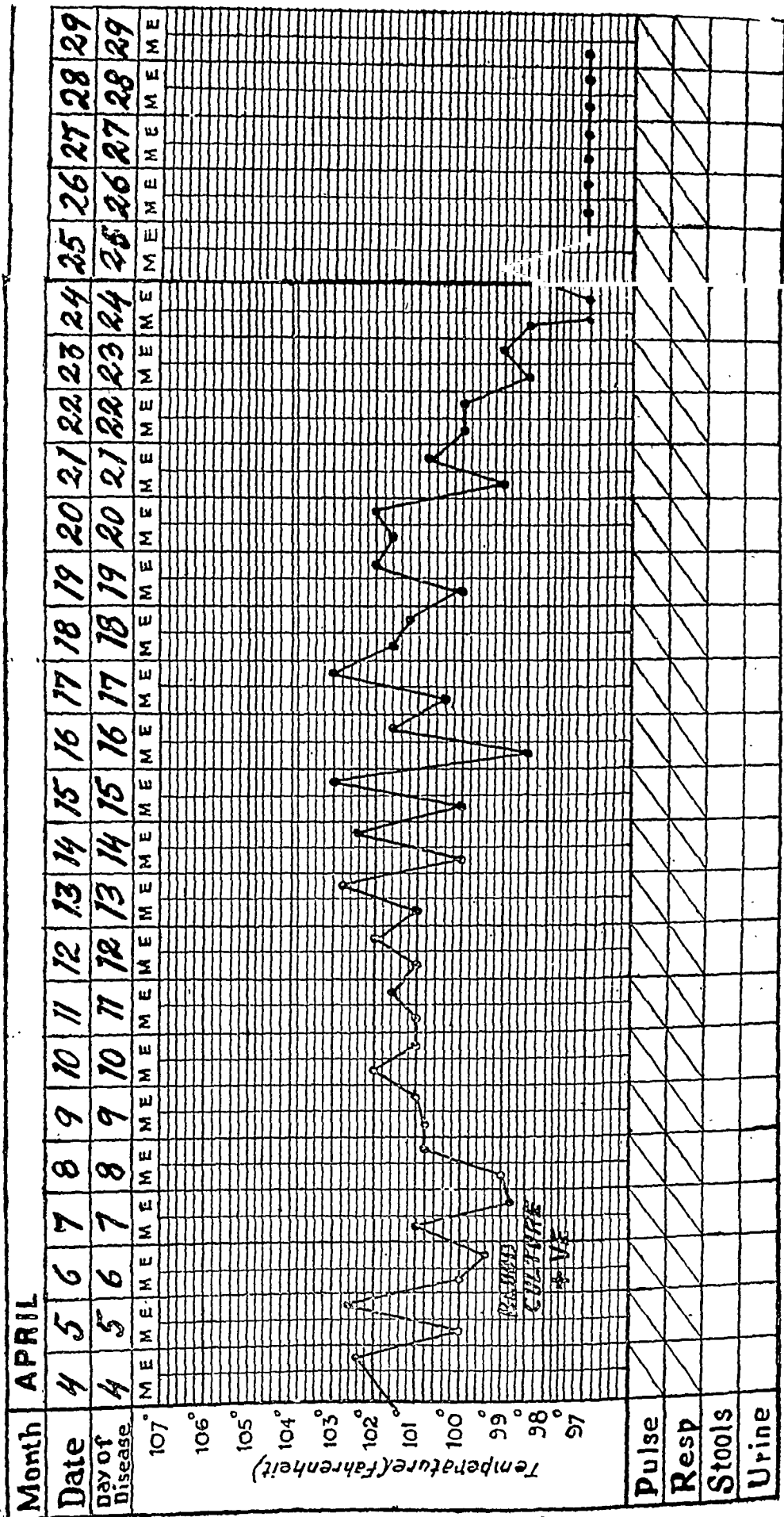
TEMPERATURE CHART 1.

King Edward VII Memorial Hospital, Bombay.

Disease—Typhoid Fever. Age—20 years.



TEMPERATURE CHART 2.
 King Edward VII Memorial Hospital, Bombay.
 Disease—Typhoid Fever. Age—25 years.



value in typhoid fever. In typhoid, which is now recognized as a primary systemic infection with incidental localization of the bacilli in the intestinal tract, orally administered 'phage is not likely to prove effective unless absorbed in sufficient amounts into the circulation.

The views regarding the efficacy of bacteriophage therapy in typhoid fever are conflicting. Many clinicians believe that the slight and temporary benefit which follows the subcutaneous or intravenous administration of the 'phage is due to induced protein-shock reaction rather than to any specific lytic action exerted on the organisms. Kolmer and Tuft (1941) have concluded that the therapeutic value of the bacteriophage has not fulfilled the original expectations and must still be determined by further clinical experience. In assessing the results reported by various observers, there is some difficulty in that many have failed to use adequate controls, there being little or no evidence to show that the medium itself in which the 'phage was grown was not responsible for the good results reported. Unsuccessful results may be obtained if sufficiently potent 'phage is not utilized.

In the state of our present limited knowledge, the type-specific Vi bacteriophage, i.e. a 'phage acting specifically against a particular type of typhoid Vi antigen, may be considered the most potent available. A combination of type-specific Vi 'phage and 'O' 'phage may prove still more effective, because such a combination in proper proportions is known to sterilize a young culture with no resistant forms left to develop subsequently.

During the present investigation, we observed several cases in which the blood-culture was obtained positive for *S. typhi* but the cases turned out to be very mild and recovered completely in a few days. Such a mild course is supposed to be common among the children, but is also sometimes met with among the adults. The typhoid fever shows great variation in its severity, and such variation has been found even in a group of persons infected from the same source. The issue, therefore, appears to depend on unknown factors in the individual resistance, and perhaps to a certain extent on the dose. Besides, many of the patients in the first week, when the disease is just started, are not very severely ill, and the condition then existing is no guide to the subsequent course. As is well known, the fatal complications of typhoid are common in the second and third weeks, and may occur even in a person in whom the disease has started mildly in the first week. Hence it is felt that strict keeping of each alternate patient as a control is essential in the true assessment of results of any therapy which does not show a spectacular effect. In the present series, among the *oral* as well as the *intravenous* cases, regular control cases were kept, and the other variables like the diet and the basic nursing treatment were kept constant as far as possible, thus rendering a comparison between the treated and control cases reasonably valid.

Among the patients given 'phage intravenously, a disturbing factor was the violent rigor, which usually lasted the better part of an hour. Knouf *et al.* (*loc. cit.*) report in their series a reduction in the severity of the reaction by distributing the administration of the 'phage over a period of about 5 hours by using the slow, continuous drip technique. We used the continuous drip method in the administration of the 'phage, yet in most of the cases the rigors obtained

were quite severe. Banerjee (*loc. cit.*) reports his observation on one successfully treated patient in whom the rigor failed to appear after previous injection of antitoxic serum. The exact cause of the rigor is debatable. It may either be due to the protein injected, or due to introduction of the endotoxin together with the 'phage preparation, or due to liberation of endotoxin after bacteriolysis *in vivo*. In the present series one control patient was injected one c.c. of autolysate (which did not contain any 'phage), and he developed a rigor very similar to that after the injection of bacteriophage. The autolysate consisted of a filtrate of 24-hour broth-culture of *S. typhi* isolated from the same patient. As the injection of autolysate was not thought of early enough, it could be tried only in one patient, and no conclusions can be drawn from the results obtained.

The work on Vi 'phage-typing of *S. typhi*, on the basis of the scheme laid down by Craigie and Felix (1947), has been started by us now, with the help of the necessary 'phage preparations received from Dr. A. Felix. In near future we hope to institute a trial of the therapeutic effect of type-specific Vi 'phage, pooled with 'O' 'phage, if necessary.

CONCLUSION.

An unselected stock bacteriophage administered to typhoid patients orally, has no therapeutic effect. The same 'phage administered intravenously appears to be of some value. The observations are being extended. In future trials it is recommended that a type-specific Vi 'phage should be used for intravenous administration, combined with an 'O' 'phage, if necessary. If in the study of the first few cases definite spectacular results are not obtained, alternate patients should be treated as controls and administered autolysate intravenously.

SUMMARY.

1. Results of treatment of 24 cases of typhoid fever with an unselected stock bacteriophage (later found to be an 'O' 'phage) are presented. Of these, 10 were treated by the oral route, and 14 by the intravenous route. Alternate patients were kept as controls, enabling a valid evaluation of the therapy. *S. typhi* was isolated from blood-culture in each patient before treatment.

2. The results of intravenous 'phage therapy appear to be sufficiently promising to warrant further trial with type-specific Vi 'phage, either alone or pooled with an 'O' 'phage.

Our thanks are due to Dr. S. G. Vengsarkar, Superintendent, The City Fever Hospital, Bombay for allowing us to investigate and treat the cases admitted under his care. We are also thankful to Dr. S. R. Pandit, Director, Pasteur Institute, Shillong for suggesting the use of 'autolysate' in control cases. We are indebted to the Indian Research Fund Association for the grant which enabled the investigation to be carried out.

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PURIFICATION OF VACCINE LYMPH BY PENICILLIN.

BY

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THERE has always been an urge amongst workers in vaccine lymph to produce a virus as free from extraneous organisms as possible, without any loss in potency. The most prevalent methods of purification of calf lymph are the employment of 0.5 per cent phenol, 0.1 per cent oil of cloves and the chloroforming process. It was to be expected that the advent of penicillin would provide fresh stimulus to the search for a purifying agent. It is understood that at least one institute engaged in the manufacture of vaccine lymph in India is at present employing penicillin in the routine process of purification of vaccine lymph. A few workers have already reported on their experience with penicillin as a purifying agent for vaccine lymph. Gohar (1946) has reported that penicillin is not superior to glycerine in the destruction of *Staphylococcal* contaminants of vaccine lymph and that the vaccine virus is sensitive to penicillin. On the other hand, Diaz Romero (1945) and Fasquelle (1947) have both concluded from their experiments that penicillin, while not inimical to vaccine virus, effects substantial reduction in the bacterial flora of vaccine lymph.

A short investigation was conducted in this Institute with a view to ascertaining whether penicillin could replace the existing method of purification, viz. the chloroforming process. The experiments were confined only to *glycerinated* lymph as otherwise they would have no bearing on the current method of preparation of vaccine lymph.

MATERIALS AND METHODS EMPLOYED.

Penicillin employed in the experiments was of a reputed make. Immediately after its solution in distilled water, it was added according to the needs of the experiment to the *glycerinated* lymph (1 in 6) which was then returned to the cold storage (-11°C). A portion of each batch of lymph employed was also purified by the usual chloroforming process, for serving as a control in the subsequent purity and potency tests. Colony-counts of the samples were made in a dilution of 1 in 10 in sterile normal saline. The agar plates poured were incubated at 37°C .

for 2 days and then at the room temperature (averaging 24°C.) for 3 days after which the number of colonies in the plates was counted. The potency tests were made on cow-calves. The requisite dilution of lymph in normal saline was made and 0.1 c.c. of the dilution was coaxed into a uniformly abraded area of 14 sq. cm. The result was read after 120 hours and the number of vesicles noted.

Experiment 1.—An initial experiment was carried out to ascertain the optimum range of penicillin concentration necessary for rendering the lymph relatively free from extraneous organisms. Calf lymph batch No. 8070 was divided into 6 lots and penicillin was added to 5 of the lots, in doses of 50, 75, 100, 125 and 150 units per c.c. respectively, while the 6th lot was chloroformed. The bacterial count was made at different intervals after treatment with penicillin. Table I summarizes the results obtained:—

TABLE I.

Bacterial count of vaccine lymph after treatment with penicillin.

Concentration of penicillin per c.c. of vaccine lymph. Batch No. 8070.	BACTERIAL COUNT PER C.C. OF VACCINE LYMPH AT DIFFERENT INTERVALS AFTER PENICILLIN TREATMENT.			
	1 day.	2 days.	4 days.	7 days.
Units—				
50 	8,500	3,800	5,000	18,000
75 	1,700	100	200	0
100 	700	200	0	0
125 	300	200	0	0
150 	100	0	0	0
Not treated with penicillin but chloroformed.	6,500	1,600	0	0

Impression will be gathered from Table I that, while penicillin in concentrations of 75 units per c.c. and above rendered the lymph practically free from extraneous organisms, in a concentration of 50 units per c.c., it effected an initial drop in the colony-count with a subsequent steep rise after 7 days. This particular lot when again tested after 1 month showed innumerable colonies per c.c. Practically all the colonies noted in these tests were those of *Staphylococcus albus*. None of the above lots produced gas in Robertson's cooked meat medium and rabbits inoculated for evidence of toxicity remained healthy for a period of over 7 days.

Potency of the above lots of lymph was tested on cow-calves 3 weeks after the process of purification in dilutions of 1 in 1,000 and 1 in 5,000.

The results are given in Table II :—

TABLE II.

Potency of lymph after penicillin treatment.

Concentration of penicillin per c.c. of vaccine lymph. Batch No. 8070.	CALF I.		CALF II.	
	1 in 1,000.	1 in 5,000.	1 in 1,000.	1 in 5,000.
Units—				
50 ...	Semi-confluent take	7 vesicles	Confluent take	12 vesicles.
75 ...	" "	7 "	" "	14 "
100 ...	Confluent take	11 "	" "	12 "
125 ...	Semi-confluent take	11 "	" "	12 "
150 ...	18 vesicles	8 "	" "	11 "
Not treated with penicillin but chloroformed.	20 "	7 "	" "	12 "

It is obvious from Table II that the potency of the vaccine virus remained unaffected by increasing concentrations of penicillin within the range adopted, the control chloroformed sample exhibiting about the same degree of potency as the rest.

Experiment 2.—In view of the results noted in Experiment 1, it was decided to confirm the findings using only one concentration of penicillin, viz. 100 units per c.c. indicated as quite suitable in that experiment. A portion of lymph batch No. 8313 was chloroformed as usual and the rest was treated with penicillin in a concentration of 100 units per c.c. The bacterial count was made 2 days, 4 days, and 7 days later as before. The results are given in Table III :—

TABLE III.

Bacterial count of vaccine lymph after treatment with penicillin.

Concentration of penicillin per c.c. of vaccine lymph. Batch No. 8313.	BACTERIAL COUNT PER C.C. OF VACCINE LYMPH AT DIFFERENT INTERVALS AFTER TREATMENT WITH PENICILLIN.		
	2 days.	4 days.	7 days.
100 units ...	Enormous number of colonies, mostly of <i>Staphylococcus albus</i> and <i>B. coli</i> .	Enormous number of colonies, mostly of <i>Staphylococcus albus</i> and <i>B. coli</i> .	Exceedingly large number of colonies, mostly of <i>Staphylococcus albus</i> and <i>B. coli</i> .
Not treated with penicillin but chloroformed.	6,000 per c.c. (No <i>B. coli</i> .)	4,500 per c.c. (No <i>B. coli</i> .)	4,000 per c.c. (No <i>B. coli</i> .)

Obviously the batch of lymph used in this experiment had an unusually large amount of bacterial flora as is indicated by the results with the chloroformed sample. The enormous number of colonies in the sample treated with penicillin is understandable as penicillin has little action on *B. coli* and the additional presence of *Staphylococcus albus* in large numbers is explained by the inactivation of penicillin by penicillinase produced by *B. coli*. In contrast, the bacterial count in the case of chloroformed sample was well below the accepted limit and in addition it is to be noted that no *B. coli* colonies were present. In the experience of this Institute which has adopted the chloroforming process for the routine purification of vaccine lymph, colonies of *B. coli* are very rarely met with in the day-to-day bacteriological examination of purified lymph.

Experiment 3.—In view of the above results the penicillin-treated portion of the batch No. 8313 was divided into 4 lots and again treated with penicillin in concentration of 250, 500 and 1,000 units respectively. The bacterial count of these samples was made 4 days, 7 days, and 1 month after treatment with penicillin.

The results are indicated in Table IV:—

TABLE IV.

Bacterial count of vaccine lymph after treatment with higher concentrations of penicillin.

Concentration of penicillin per c.c. of vaccine lymph.		BACTERIAL COUNT PER C.C. OF VACCINE LYMPH AT DIFFERENT INTERVALS AFTER TREATMENT WITH PENICILLIN.		
Batch No. 8313.		4 days.	7 days.	1 month.
Units—				
250	Numerous colonies, mostly of <i>B. coli</i> and <i>Staphylococcus albus</i> .	Numerous colonies, mostly of <i>B. coli</i> and <i>Staphylococcus albus</i> .	Numerous colonies, mostly of <i>B. coli</i> and <i>Staphylococcus albus</i> .
500	Many <i>B. coli</i> colonies only.	Many <i>B. coli</i> colonies and a few <i>Staphylococcus albus</i> colonies.	Many <i>B. coli</i> colonies and numerous <i>Staphylococcus albus</i> colonies.
1,000	Many <i>B. coli</i> colonies only.	Many <i>B. coli</i> colonies only.	Many <i>B. coli</i> and <i>Staphylococcus albus</i> colonies.

It will be observed from Table IV that in the presence of *B. coli* even the high concentration of 1,000 units per c.c. was not productive from the standpoint of purity standards, and that increasing concentrations of penicillin suppressed *Staphylococci* to a larger extent and for a longer period of time.

Experiment 4.—The results in Experiments 2 and 3 which were conducted with a lymph batch having a preponderance of *B. coli* obviously could not alone lead to a fair appraisal of the efficacy of penicillin in the purification of vaccine lymph. Further, the results obtained in Experiment 1 justified further trials with a larger number of batches of lymph. An experiment was, therefore, carried out on four batches using two concentrations of penicillin for each batch, viz. 100 and 150 units per c.c. respectively. A portion of each batch was also chloroformed as usual to act as a control. The bacterial counts obtained with these samples are given in Table V :—

TABLE V.

Bacterial count of vaccine lymph after treatment with penicillin.

Number of lymph batch.	Concentration of penicillin per c.c. of vaccine lymph.	BACTERIAL COUNT PER C.C. OF VACCINE LYMPH AT DIFFERENT INTERVALS AFTER TREATMENT WITH PENICILLIN.	
		4 days.	7 days.
8101 ...	100 units ...	100	0
	150 „ ...	0	0
	Chloroformed ...	4,200	2,000
8124 ...	100 units ...	0	0
	150 „ ...	0	0
	Chloroformed ...	8,000	5,200
8303 ...	100 units ...	0	0
	150 „ ...	0	0
	Chloroformed ...	5,900	5,500
8304 ...	100 units ...	Large number of colonies (no <i>B. coli</i>).	Large number of colonies (no <i>B. coli</i>).
	150 „ ...	„ „	„ „
	Chloroformed ...	900	1,000

The results with penicillin in the case of batches Nos. 8101, 8124 and 8303 appear both striking and spectacular; while those with batch No. 8304 are puzzling, particularly as there was no evidence of *B. coli* in its bacterial flora. Further, this lymph when tested was found to be as distinctly alkaline as the others. All the above lots, as also a sample of each exposed to the room

temperature (averaging 24°C.) for 7 days, were tested for potency on calves, 3 weeks after penicillin treatment. Table VI summarizes the results obtained:—

TABLE VI.
Potency of vaccine lymph after penicillin treatment.

Number of lymph batch.	Concentration of penicillin per c.c. of vaccine lymph.	VESICLES ON CALF I.				VESICLES ON CALF II.			
		Exposed to the room temperature for 7 days.		Unexposed.		Exposed to the room temperature for 7 days.		Unexposed.	
		1 in 1,000	1 in 5,000	1 in 1,000	1 in 5,000	1 in 1,000	1 in 5,000	1 in 1,000	1 in 5,000
8101 ...	100 units ...	25	20	SC	SC	13	10	C	C
	150 „ ...	SC	20	C	SC	SC	SC	C	C
	Chloroformed ...	SC	SC	C	SC	SC	SC	C	C
S124 ...	100 units ...	SC	25	C	C	C	C	C	SC
	150 „ ...	SC	18	C	C	SC	20	SC	21
	Chloroformed ...	SC	20	C	C	25	20	SC	18
S303 ...	100 units ...	0	1	7	5	7	0	SC	7
	150 „ ...	3	2	7	4	3	2	SC	8
	Chloroformed ...	2	1	4	3	5	1	SC	4
S304 ...	100 units ...	8	3	SC	10	SC	8	C	SC
	150 „ ...	6	0	10	5	SC	13	C	20
	Chloroformed ...	6	2	C	8	SC	5	C	SC

C = Confluent take.

SC = Semi-confluent take.

Table VI clearly indicates and confirms the findings in Experiment 1, that penicillin in concentrations tested did not affect the potency of the virus, there being no significant difference in results in the dilution tested between the lots treated with 100 and 150 units per c.c. respectively and the chloroformed lot, under similar conditions of storage.

Experiment 5.—With a view to confirming the results obtained in the various purity tests in the previous experiments, it was decided to repeat after longer intervals the bacterial count in the case of all the samples which had given striking results soon after treatment with penicillin. This seemed particularly necessary in view of the trends towards increasing bacterial counts with

subsequent tests, shown by those samples which were treated with too inadequate a concentration of penicillin or had *B. coli* in their bacterial flora. The confirmatory tests were carried out 6 to 8 weeks after treatment with penicillin. The results are summarized in Table VII :—

TABLE VII.

Bacterial count of vaccine lymphs 6 to 8 weeks after treatment with penicillin.

Number of lymph batch.	Concentration of penicillin per c.c. of vaccine lymph.	BACTERIAL COUNT PER C.C. IN PREVIOUS TESTS.		Interval between the present test and the date of treatment with penicillin, weeks.	Bacterial count in the present test.
		4th day.	7th day.		
8070	75 units ...	200	0	8	Numerous colonies, mostly of <i>Staphylococcus albus</i> .
	100 " ...	0	0	8	" "
	125 " ...	0	0	8	" "
	150 " ...	0	0	8	" "
	Chloroformed	0	0	8	200 per c.c. (<i>Staphylococcus albus</i>).
8101	100 units ...	100	0	6	Numerous colonies, mostly of <i>Staphylococcus albus</i> .
	150 " ...	0	0	6	" "
8124	100 " ...	0	0	6	" "
	150 " ...	0	0	6	" "
8303	100 " ...	0	0	6	" "
	150 " ...	0	0	6	" "

It is clear from Table VII that all the batches which gave the impression of having been rendered totally free from extraneous organisms had really not been so purified as is evidenced by the results in tests conducted 6 to 8 weeks later.

Experiment 6.—The foregoing results clearly indicated that the spectacular results obtained soon after treatment with penicillin might only be due to free penicillin present in the lymph, exerting its action in the agar medium used for the bacterial count, and not to actual purification of lymph. To investigate this it was decided to repeat the experiments, using penicillinase at the time of putting up the test for the bacterial count. For this purpose a culture of *B. coli* was

inoculated into ordinary nutrient broth and incubated at 37°C. for 2 days. The broth culture after a preliminary coarse filtration was filtered through an L₃ candle. The filtrate was tested for sterility and found sterile. On testing the filtrate for penicillinase content, it was found that approximately 1 c.c. of the filtrate was required to neutralize 100 units of penicillin.

Lymph batch No. 8171 was divided into 5 lots and of these one lot was purified by the usual chloroforming process. The remaining 4 lots were treated with penicillin in concentrations of 100, 500, 1,000 and 5,000 units per c.c. respectively. All the lots were tested for colony-count 5 days and 12 days after penicillin treatment. The penicillin-treated lymphs were also tested by the addition to the agar plate of the requisite quantity of penicillinase for complete inactivation of the penicillin content in the actual amount of undiluted lymph that went into the agar plate. Care was taken to thoroughly mix the contents as soon as the melted agar was poured into the plates.

The results are summarized in Table VIII :—

TABLE VIII.

*Bacterial counts of penicillin-treated lymphs with and without penicillinase.**

Concentration of penicillin per c.c. of vaccine lymph.	BACTERIAL COUNT PER C.C. OF VACCINE LYMPH AT DIFFERENT INTERVALS AFTER TREATMENT WITH PENICILLIN.			
	5 days.		12 days.	
	Without penicillinase.	With penicillinase.	Without penicillinase.	With penicillinase.
Batch No. 8171.				
Units—				
100 ...	0	Enormous number of colonies.	0	Enormous number of colonies.
500 ...	0	"	0	"
1,000 ...	0	"	0	"
5,000 ...	0	"	0	"
Not treated with penicillin but chloroformed.	1,200	...	1,500	...

* The penicillinase containing filtrate when tested was found sterile on each occasion.

It is obvious from these results that even such a high concentration of penicillin as 5,000 units per c.c. failed to purify lymph. It is also now quite clear that all the spectacular results observed soon after treatment with penicillin were not due to actual purification of lymph but to the action in the agar medium of penicillin carried over in the quantum of lymph tested. With the passage of time free penicillin in the treated lymphs must have completely disappeared

or very considerably reduced, which would explain the growth of innumerable colonies in tests conducted 6 to 8 weeks later.

CONCLUSIONS.

An analysis of the results obtained in the foregoing experiments leads to the following conclusions regarding the use of penicillin in the purification of vaccine lymph :—

1. Penicillin in concentrations tested exerts absolutely no deleterious effect on the potency of the virus. Even under adverse conditions of storage there is no deterioration beyond that which is to be expected during the period of such storage even without penicillin.

2. Penicillin even in as high a concentration as 5,000 units per c.c. does not purify glycerinated calf lymph. It seems evident that all the favourable reports on penicillin as a very suitable purifying agent for the routine purification of *glycerinated* calf lymph may be based only on results obtained soon after treatment with penicillin; no penicillinase being used in the tests and the initial tests not being followed up by subsequent ones after much longer intervals.

These experiments were conducted with *glycerinated* calf lymph and as such the conclusion pertaining to the unsuitability of penicillin for purification has only reference to *glycerinated* calf lymph. However, whatever the diluent used for calf lymph it is obviously extremely desirable that in experiments of this nature penicillinase should be employed in the purity tests so that a proper appraisal of the utility of penicillin can be made.

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THE AMELIORATION OF SYMPTOMS OF FLUOROSIS BY ALUMINIUM SALTS.

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SINCE it was reported (Smith, Lantz and Smith, 1931) that the ingestion of water containing fluorides causes a dental dystrophy known as 'mottled enamel', considerable work has been done on this subject. It has been shown (Cumming, 1935; Dean, 1936) that the incidence and severity of the mottling correlates with the concentration of fluorine in the drinking water. Continued ingestion of fluoride-bearing water has been shown (Shortt, Barnard, McRobert and Nayar, 1937) to cause disturbances in the skeletal system similar to those described by Roholm (1937) among workers in cryolite factories.

Research workers have been endeavouring, on the one hand, to devise suitable methods of removing fluorides from drinking water and, on the other hand, to seek out antidotes for this poison.

The observation that animals on a low-calcium diet are more susceptible to fluorosis (Ranganathan, 1941) than the animals on an adequate diet led to the investigation of the possibility of administering calcium salts to ameliorate the condition. Ranganathan (1944) found that calcium salts do exert a beneficial action and that there is not much difference between one salt of calcium and another. Pillai, Rajagopalan and De (1944) have reported the beneficial action of foods (such as milk and bone meal) which are rich in calcium. Pandit *et al.* (1940) came across two adjacent villages whose inhabitants showed a marked difference in the incidence of fluorosis. This difference could not be explained on the basis of the fluoride content of the drinking water or the calcium content of the diet. The only major difference was that the diet consumed in the

affected village was poor in vitamin C. From a set of animal experiments conducted on the monkey (*Macacus radiatus*) Pandit and Rao (1940) concluded that monkeys which did not receive vitamin C in the diet showed greater adsorption of fluorine and also showed more marked radiological changes than the monkeys which received an adequate amount of vitamin C. Majumdar and Ray (1946) concluded from his experiments on hill bulls that even when a diet contains adequate amounts of calcium and phosphorus the relief afforded is only temporary and is useless to save a victim from the effects of prolonged high intake of fluorine.

In their attempts to compare the toxicities of different compounds of fluorine, various workers have fed sodium fluoride, calcium fluoride and cryolite to experimental animals. Smith and Leverton (1934) found that whether we take into consideration the effect on the teeth, the growth rate or the mortality, a much larger dose of fluoride is required when it is given in the form of cryolite. Kempf, Greenwood and Nelson (1937) found that rats receiving 0.025 per cent fluorine in their diet developed severe mottling but that rats receiving the same amount of fluorine along with 0.396 per cent aluminium sulphate had normal teeth. Sharpless (1936) fed aluminium chloride to rats receiving fluorine at 0.025 and 0.1 per cent of their diet and found a marked improvement in the condition. The amelioration effected by feeding aluminium sulphate was superior to that of calcium lactate, calcium phosphate, magnesium hydroxide, boric acid, borax, bone meal, clay and aluminium powder according to the experiments of Marcovitch and Stanley (1942) who came to the conclusion that aluminium sulphate was most effective in combating the decrease in rate of growth of rats receiving 0.2 per cent sodium fluoride in their diet. Likewise, alum was found to protect rabbits against lethal doses of sodium fluoride. Majumdar and Ray (1946) were the first to record that the administration of aluminium phosphate prevented the formation of exostoses in bulls receiving fluorine in their diet. Since the problem as it affects the human and cattle population is concerned mostly with the need to prevent formation of these troublesome skeletal changes, it appeared desirable to investigate an antidote (like alum) which has shown a capacity to prevent the formation of such exostoses. Advantage was taken of some rat-feeding experiments that were in progress (to study the effectiveness of an activated carbon prepared by us for removal of fluorides from water) to run a preliminary experiment to confirm the ameliorative effect of alum.

EXPERIMENTAL.

Young albino rats (6 weeks old), weighing between 35 g. and 40 g., were divided into three groups of six animals. Each group contained an equal number of males and females. The stock diet, devised to reproduce as far as practicable the diet consumed in the endemic areas of the Madras Presidency, consisted of powdered ragi (*Eleusine coracana*) 45.9 per cent, bajra (*Pennisetum typhoides*) 45.9 per cent, tugar dhal (*Cajanus indicus*) 3.6 per cent, Bengal gram (*Cicer arietinum*) 3.6 per cent, skim milk powder 0.05 per cent, common salt 0.1 per cent, and gingelly oil 1.0 per cent. This diet contained about 9 per cent protein and 0.5 per cent calcium (as CaCO_3). The rats were given 5 g. of the diet to start with, the quantity being gradually increased at the rate of one gramme per week till a

level of 10 g. was reached. Every week, fresh brinjals (*Solanum melongena*) and amaranthus leaves (*Amaranthus gangeticus*) were cut and fed to the rats to make up 10 per cent of the level of the week's diet. Each batch of the diet mixture was analysed for its protein and calcium content.

The rats of the first (*control*) group received their diet mixed up in water, while the rats of *fluorine-alone* group received one milligram fluorine (added as sodium fluoride) in the diet. The rats of the third (*fluorine plus alum*) group received 1 mg. fluorine as NaF and 200 mg. aluminium added as a solution of the salt $[\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}]$. The rats were weighed at the end of each week and their teeth carefully examined.

RESULTS.

The rate of growth in all the groups is almost identical (see Table I).

TABLE I.

Average increase in weights.

Number of weeks.	Control, i.e. no fluorine.	Fluorine group.	Fluorine plus alum.
1	4.1	4.3	2.8
2	4.8	5.3	4.3
3	7.8	7.3	5.9
4	7.1	7.1	6.1
5	9.8	9.0	10.6
6	8.0	9.1	8.0
7	8.0	8.0	8.5
8	10.9	11.3	11.5
9	6.3	5.6	5.5
10	4.8	3.8	5.1
11	8.6	6.5	9.5
15	11.1	13.5	13.7
Average ...	6.08	6.05	6.1

The teeth of the rats in the *control* group continued to remain normal till the conclusion (15 weeks) of the experiment. In the group receiving *only fluorine*, the lower incisors began to show bleaching in the second week and were completely bleached to a chalky-white in the course of five weeks. From about that time, the upper teeth started showing signs of bleaching, while the lower teeth showed

broken or eroded tips. About the ninth week of the experiment, the upper teeth in 3 out of 6 animals began to grow inwards and were also completely bleached to a dull chalky-white colour. Thereafter, other changes such as erosion of the tips, missing of the teeth as well as malformations were evident. By about the second week the rats in the group receiving *fluorine as well as alum* showed some signs of bleaching of the lower teeth but only 50 per cent of the animals were affected as against all the animals in the fluorine group. This condition slowly progressed and faint striations were noticeable about the sixth week, but it is noteworthy that the more severe symptoms such as erosion of the tips or malformations never occurred in any rat of this series, even at the end of the fifteen-week period.

X-ray pictures of the skeletons of the rats failed to reveal any significant differences between these three groups.

At the conclusion of the experiment the rats were sacrificed, and the corresponding femurs and their epiphysis were analysed for fluorine (ashing in presence of a fixative and distillation with silica and sulphuric acid). The results (see Table II) show that the rats receiving alum in addition to fluorine have stored much less fluorine in their bones as compared to the rats receiving fluorine alone.

TABLE II.

Fluorine content of the bones of the experimental animals.

Group.	Number of animals taken for analysis.	Femur, per cent.	Epiphysis, per cent.	PERCENTAGE INCREASE IN F OVER CONTROL.	
				Femur.	Epiphysis.
Control	3	0.114	0.201
Fluorine group	3	0.381	0.585	234.2	240.5
Fluorine <i>plus</i> aluminium ...	3	0.312	0.495	173.7	146.3

DISCUSSION.

The amelioration of the symptoms of fluorosis, particularly the more severe symptoms, by the administration of aluminium sulphate in the diet of experimental rats has been confirmed by these experiments.

The absence of variations in the rate of growth in the three groups is not surprising when we consider the low level of fluorine administered (0.01 per cent of diet). In two other sets of experiments where we had administered fluorine at the same levels, we have not been able to notice any difference in the rate of growth between the animals receiving fluorine and the control. Sharpless (*loc. cit.*) also finds that by feeding fluorine at a level of 0.025 per cent in the diet, the rate of growth is not depressed and that it is necessary to feed fluorine at 0.1 per cent of the diet to get a perceptible decrease

The absence of any visible changes in the skiagrams is not of any significance since the rats receiving only fluorine also showed no perceptible changes.

Our knowledge of the detailed mechanism of the several effects of fluoride ingestion is not complete. It may, however, be tentatively suggested that as soon as the fluorides get into the blood stream, they cause a depletion of the blood calcium due to the formation of calcium fluoride or of fluorapatite which then gets deposited on the long bones. If the diet is not adequate in calcium, it is conceivable that the skeleton is drawn upon for the requirements of calcium with the consequence that the bone becomes weak (Majumdar and Ray, 1943). In any case, the fluorine does not appear to affect other organs until the teeth and bone are nearly saturated. It may even be suggested that the skeletal storage of fluorine is a defensive mechanism of the body. Brandl and Tappeiner (1937, quoted by Roholm, *loc. cit.*) record that there is a time-lag between the administration of fluorine and its appearance in the urine. In other words, until the capacity of the skeletal structures is nearly exhausted fluorine does not persist in the blood and find its way into the kidney. On this hypothesis it would be reasonable to expect that the administration of calcium salts could spare the skeleton or reduce the more acute symptoms caused by administration of heavy doses of fluorine but it would not be expected to prevent the formation of exostoses. Majumdar *et al.* (*loc. cit.*) found that administration of calcium phosphate did not prevent exostoses in bones of cattle receiving fluorine.

The mechanism of the protective action of aluminium is not clear. Majumdar finds that the proportion of fluorine excreted through the intestines is only slightly increased in the animals receiving aluminium supplement and that this small increase cannot explain the protective action. Sharpless suggests that the protective action is probably due to the formation of a slightly dissociated salt of calcium and aluminium. Some evidence for this view has been obtained in our experiments on the possibility of using calcium aluminate to remove fluorides from water. Whatever be the exact mechanism of the beneficial action of aluminium supplement, the fluorine content of the bones can be expected to give an indication of the amount of fluorine metabolized. The reduction in the amount of fluorine stored in the bones of the animals receiving aluminium sulphate is, therefore, significant.

It is proposed to undertake further experiments, particularly with animals liable to form exostoses on the long bones, and conduct detailed metabolic studies to determine the precise rôle of aluminium in ameliorating the symptoms of fluorosis.

SUMMARY.

This work has confirmed the ameliorating effect of aluminium salts in fluorosis induced in albino rats and has shown that the skeletal storage of fluorine is appreciably reduced by the presence of aluminium in the diet.

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STUDIES ON ANÆROBIC WOUND INFECTION.

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THOUGH cases of gas-gangrene and tetanus were known to occur even in the days of Hippocrates, the real scientific study of the anærobic wound infection began with the birth of bacteriology in the latter part of the 19th century. In the earlier days, because of the faulty technique employed in the isolation of anærobes, the knowledge of these anærobes was shrouded in mystery and a great confusion existed. It is in this confused state that the surgeons faced the first world war, with the result that in 1914 the incidence of gas-gangrene in the wounded, amongst the British Expeditionary Force, amounted to over 12 per cent. According to Ziessler (1940, quoted by Altemeir and Fruste, 1947) 100,000 German soldiers died of this complication during the first world war. This at once transformed the problem of *clostridial* wound infection into one of highest importance and served as an effective stimulus to undertake a thorough study of the anærobes and their rôle in the wound infection. Introduction of McIntosh Fildes' jar was a step forward, which made it possible for the bacteriologists to obtain surface growths of anærobes required for the isolation of the strains in pure cultures. Thanks to the combined efforts of the surgeons and the bacteriologists in the first world war, much of the obscurity surrounding anærobic wound infection was unravelled and gas-gangrene became established as a clinical manifestation, produced by a variety of pathogenic anærobes, either alone or in combination. In spite of the extensive work that followed the impetus, given by the first world war, which fortunately did not end with it, studies on anærobes were continued further. Many of the problems, such as the pathogenesis of gas-gangrene, the immunity reactions that follow the infection, await solution and require more work. With the advent of the second world war in 1939, this problem again assumed major importance and attempts were made in this Laboratory to study the methods of cultivation of anærobes and it became possible to isolate and identify few strains of anærobes with relative ease. Encouraged by the preliminary results,

it was decided to undertake the study of anaerobic wound infection, in view of the fact that the incidence of *clostridia* in wound infection varies according to the geographical conditions. The knowledge of the organisms responsible for gas-gangrene in this part of the world was considered essential should India get enveloped in the world war.

MATERIAL.

This was made available from various sources, such as exudates from the deep portions of fresh wounds, wounds in surgical wards, cases of tetanus or from autopsy cases in which a suspicion of anaerobic infection was entertained. In addition to this, samples of manure and road-side dust round the vicinity of the hospital compound were tested for the presence of anaerobes. Similarly, samples of human and guinea-pig stools were submitted to bacteriologic examination in order to isolate strains of *Cl. welchii* to be studied in comparison with the strains of *Cl. welchii* isolated from the cases of gas-gangrene. In all 210 samples were investigated for the presence of anaerobes. Table I gives their distribution according to the source :—

TABLE I.

Distribution of anaerobes according to sources.

Source of material.					
Fresh wounds	47
Surgical cases	84
Autopsy material	20
Samples of manure and road-side dust	15
Samples of human stool	15
Samples of guinea-pig stools	8
Miscellaneous	12
TOTAL					210

As in anaerobic wound infections, the type of injury has some bearing on the genesis of *clostridial* infection ; in Table II is shown the type of injury in 47 cases of fresh wounds studied for their anaerobic flora :—

TABLE II.

Types of fresh wounds.

Incised wounds	2
Contused lacerated wounds involving skin and subcutaneous tissue.	22
Lacerated wounds involving muscles	9
Compound fractures	11
Punctured wounds	3
TOTAL					47

Similarly, amongst the 210 samples was material collected from 25 clinically diagnosed cases of gas-gangrene. Table III gives the type of injury in these 25 cases, while in Table IV is shown the arrangement of these cases according to the site and outcome of the infection:—

TABLE III.
Type of wounds.

Crushed injuries with compound fractures	14
Anærobic infection secondary to the pre-existing gangrene of other type.			3
Brought about by traumatic peritonitis and intestinal perforation			2
Post-natal	2
Punctured wounds	2
Not known	2
TOTAL			25

TABLE IV.
Site and outcome of the infection.

Site.	Head and face.	Superior extremity.	Thorax.	Abdomen.	Buttock.	Thigh.	Leg and foot.	Total.
Recovered	...	3	1	...	4	8
Expired	...	2	...	6	3	1	1	13
Could not be traced	...	1	3	4

TECHNIQUE.

The material from the various sources mentioned in Table I consisted either of (i) wound exudates collected by means of sterile swabs from the deeper portions of the wounds, (ii) small bits of tissues, e.g. muscle fibres, and (iii) samples of stools, manure and road-side dust collected in a sterile container. After subjecting the material to the preliminary smear examination, a saline suspension was prepared by thoroughly mixing the material in 10 c.c. of sterile normal saline. This suspension was divided into two parts, one of which was heated either at 60°C. for 30 minutes or at 80°C. for 20 minutes. The unheated saline suspension was cultured in nutrient broth and in cooked-meat media tubes. The latter medium as it supported the growth of both the aerobes and anaerobes acted as a stock material to which one could return if required. The inoculated tubes were incubated anaerobically at 37°C. At the end of 24 hours of incubation the cultures were examined. The heated saline suspension was seeded in the cooked-meat media tubes which were heated just prior to the inoculation in water bath for 20 minutes, to get rid of the dissolved oxygen. Particular attention was paid to inoculate large inocula consisting of pipettefuls of the suspension. These tubes were incubated anaerobically. Anaerobiosis was achieved by McIntosh Fildes' jar. The hydrogen was supplied from the hydrogen cylinder or from Kipp's apparatus. A test-tube containing equal volumes of (i) N/10 NaOH 6 c.c. water to 100 c.c., (ii) 3 c.c. of 0.5 per cent methylene blue, and (iii) 6 per cent glucose, boiled till it became colourless, was used as an indicator for anaerobiosis. The incubation time for anaerobic cultures varied from 24 to 96 hours depending upon the type of organisms. At the end of the requisite incubation period the jar was opened and the culture tubes taken out. Changes produced in cooked-meat media were noted as also the organism grown in the tubes. Sub-cultures were made on glucose (1 per cent) agar and glucose 1 per cent blood-agar slopes or plates, as a routine, slopes were preferred to the plates. In some of the glucose-agar tubes the material was inoculated in the water of condensation. This procedure was useful in isolating organisms like *Cl. tetani* where surface growths show the tendency to spread as a film over the entire surface of the medium. Two of the culture tubes were incubated aerobically and the rest anaerobically, the period of incubation varying from 24 to 96 hours depending upon the organisms. At the end of the required incubation period the characters of the surface growths of the organisms grown were studied and ascertained by smear examination. By fishing out the characteristic colonies sub-cultures were made in the previously heated cooked-meat media tubes. These were incubated anaerobically for the requisite incubation period. The procedure of alternate growth in liquid and solid media was repeated several times to ensure the purity of the strain. The anaerobic character of the strain was ascertained by plating it on blood-agar and agar slopes and incubating some of these tubes aerobically and the rest anaerobically. Absence of the growth in the tubes incubated aerobically indicated that the strain under study was an anaerobic one.

The isolated anaerobic strain was identified by employing several criteria such as morphology, motility, colony-characters and biochemical reactions. Animal pathogenicity was tested by injecting a young culture of the strain in a white mouse. Adopting this procedure it became possible to isolate 87 strains of

anaerobes from the 210 samples. The incidence of these strains in relation to the source from which they were isolated is shown in Table V. Table VI gives the incidence of anaerobes isolated from 25 cases of gas-gangrene studied :—

TABLE V.

Incidence of the strains isolated.

Source of material.	<i>Cl. welchii.</i>	<i>Cl. tetani.</i>	<i>Cl. sporogenes.</i>	<i>Cl. sphenoides.</i>	<i>Cl. histolyticum.</i>	<i>Cl. septicum.</i>	Unidentified.	Total.
Surgical cases ...	9	12	19	...	3	5	1	...
Fresh wounds	1	5
Autopsy material ...	4	3	10	...	1	4
Samples of manure and road-side dust.	2	2
Samples of human and guinea-pig stools.	6
Miscellaneous
TOTALS ...	10	16	36	2	4	9	1	87

TABLE VI.

Incidence of anaerobes isolated from the cases of gas-gangrene.

<i>Cl. welchii</i> alone	10
<i>Cl. welchii</i> with <i>Cl. septicum</i>	3
<i>Cl. septicum</i> alone	6
<i>Cl. histolyticum</i> alone	4
No organisms	2
TOTAL	25

DISCUSSION.

1. *Anaerobic flora of the fresh wounds.*—It has been suggested that the anaerobes gain entry into the wound at the time of its infliction. Clostridial contamination of fresh accidental wounds has been the subject of study by various workers. Altmeir and Gibbs (1944) in their study of 99 wounds found *Cl. welchii* in 39.4 per cent and other clostridia in 6 per cent. They ascribe the higher incidence of the anaerobes in their series to the fact that the wounds studied were extensive and visibly dirty. Palaski, Meleney and Spaeth (1941) investigated the bacterial flora of 200 wounds of which 102 were clean and 98 were dirty wounds. They reported the presence of *Cl. welchii* in 23 per cent of the whole series mentioning that the anaerobes contaminated the dirty wounds three times more frequently than the clean wounds. Miles *et al.* (1940) found *Cl. welchii* in 34.7 per cent, *Cl. septicum* in 2.2 per cent and other clostridia in 8.7 per cent in 46 cases investigated from the war casualties in which the samples were taken within three days of the infliction of the wound. In view of these findings 47 cases of fresh accident (Table I) brought to the casualty department of the King Edward VII Memorial Hospital, Bombay, were studied for the anaerobic flora. These were cases of injuries caused by motor, tram, railway accident, etc. This group, as will be seen from Table II, contained every type of injury. Largest number in this series was of contused lacerated wounds and compound fractures.

Samples obtained from these 47 cases showed the presence of only 6 strains of anaerobes (Table V), 5 were strains of *Cl. sporogenes* and one was of *Cl. tetani*. The main source of the anaerobic wound infection is soil and hence the incidence of anaerobes in wound is bound to vary according to the content of anaerobes in the contaminating soil. Reference to Table I shows that in the present study 15 samples of manure and road-side dust round about the vicinity of the King Edward VII Memorial Hospital, Bombay, were collected and investigated for the presence of anaerobes. Only two strains of *Cl. sporogenes* were isolated from these samples. This would explain the low incidence of anaerobes in the cases of fresh wounds investigated in the present study. MacLennan (1943) has published interesting observations in this connection. He was able to isolate 22 strains of *Cl. welchii* from 26 samples of soil from the cultivated lands of African desert, 6 strains of *Cl. welchii* out of 24 samples from the soiled areas and only one strain of *Cl. welchii* in 41 samples collected from unsoiled areas. He also noted that when the fighting moved from the Western Desert to the more cultivated areas of Tripolitania and Tunisia, the incidence of anaerobic wound infection increased. In a city like Bombay, the chances of contamination of the wound by cultivated soil are less. This accounts for the lower incidence of anaerobic wound infection in the civil practice.

2. *Cases of clostridial wound infection.*—Broadly speaking, cases of clostridial wound infection can be grouped as (a) cases of tetanus and (b) cases of gas-gangrene.

(a) *Cases of tetanus.*—In the present series 11 definite cases of tetanus were studied with a view to isolating *Cl. tetani*. Only in 4 cases *Cl. tetani* could be isolated. Some of the wounds in these cases of tetanus were either healing or had practically healed. In these cases it was not possible to collect material from the

deeper portions and this may account for the low percentage of positive cultures obtained from cases of tetanus. Out of the 16 strains of *Cl. tetani* isolated in the present series (Table V) 9 strains of *Cl. tetani* were isolated from wounds in which no suspicion of infection by *Cl. tetani* was entertained. It appears that *clostridia* may be present in the wounds merely as contaminating organisms. Thus, De Waal (1943) in his study of 708 wounds found the presence of anaerobes in 58 cases out of which only three developed signs of *clostridial* infection. Similar are findings of Fleming (1915), Robertson (1941) and Manson (1932).

(b) *Cases of gas-gangrene.*—In the period of about 3 years, during which the work of isolation of anaerobes was carried on, it was possible to obtain material only from 25 cases of clinically diagnosed gas-gangrene. This fact is in accordance with the low incidence of the anaerobic flora of both the fresh wounds and that of the road-side dust and manure met with in the study (referred to in the discussion above). Two main factors concerned in the genesis of gas-gangrene are: (i) the presence of pathogenic anaerobes and (ii) conditions favourable for the development of proliferating vegetative forms of these organisms in the wound. Tissue debility or the presence of necrosed tissue, particularly muscle fibres under high tension, produces suitable conditions for anaerobic requirement needed for the growth of these organisms.

Table III shows that 14 out of 25 cases occurred as a complication of crushed injury and compound fractures. This association of crushed wounds and compound fractures with *clostridial* infection is also well borne out by the findings of other workers. According to Callender and Coupal (1929, quoted by Altemeir and Fruste, 1947), in American Expeditionary Forces, there were 1,329 cases of gas-gangrene amongst 25,272 cases of fracture of bones. In Miller's (1932) series 60.5 per cent of cases collected from civilian life occurred in individuals who had fractured bones.

In two cases in the present study *clostridial* infection resulted from traumatic rupture of intestine. These cases had an accident and showed the presence of marked haematoma in the abdominal cavity. Clinically, signs of gas and fluid were detected in the peritoneal cavity. On exploratory laparotomy these findings were confirmed and it was found that small intestine had ruptured at many places. The autopsy examination in addition to above showed foamy liver and spleen. From the peritoneal exudate and the liver *Cl. welchii* was isolated. These cases present an interesting feature as regards the source of infection which appears to be autogenous. It is a well-known fact that *clostridia* are present in the alimentary tract and given a chance, as in these two cases, may cause the infection. In two other cases the infection followed an abortion. These patients were admitted for high temperature with the history of an abortion in a moribund state. Autopsy examination revealed ecchymotic patches all over the body. There was no evidence of any external injury in them. Liver and spleen showed typical foamy appearance. The peritoneal exudate and the liver and in one the products of gestation yielded the growth of the pathogenic anaerobic organism. These cases merit special mention because the *clostridial* infection after delivery is not common. Hill (1936) in his review of the literature could only find 84 such cases to which he added 30 of his own study. In view of Butler's (1942) work, it

is likely that the infection in the two cases referred to, may be due to the anaerobes normally present in the genital tract of women.

From Table IV it appears that the location of the wound has some bearing on the genesis of *clostridial* infection. The muscular areas, such as thighs, calf and buttock, seem to be well suited. Twenty-one out of 25 cases under study were located in these regions, while not a single case was met with where the infection occurred in the region of the scalp and face or thorax. Miller (*loc. cit.*) in his collection of 607 cases collected from civilian life reports the frequency of site of infection as stated in Table VII:—

TABLE VII.

Regional frequency of cases of gas-gangrene in Miller's series.

	Per cent.
Head and neck	2.4
Trunk and genitalia	16.3
Upper extremities	22.6
Lower extremities	47.6
Extremities either left or right	11.3

3. *Bacteriologic findings in cases of gas-gangrene studied.*—Almost all of the samples collected from these 25 cases of gas-gangrene showed the presence of aerobic organisms such as *streptococci*, *staphylococci*, *diphtheroids*, etc. American workers in the last war noted that the association of pathogenic *clostridia* with *Streptococcus pyogenes* and *Staphylococcus aureus* produced a more virulent infection than that caused by pathogenic anaerobes alone. It is likely that the presence of aerobes may have symbiotic and synergic effect because of the lowering of the oxidation-reduction potential necessary for the germination of spores of anaerobic organisms.

Reference to Table VI indicates that in 23 out of 25 cases of gas-gangrene the responsible anaerobes were isolated. The clinical diagnosis in the two cases, which yielded negative results, was made on roentgenologic evidence of the presence of gas. This roentgenologic test for the diagnosis of gas-gangrene infection was first introduced during the last war. It was thought that this would enable the surgeons to diagnose gas-gangrene long before clinical signs had developed. Later studies have shown that the gas may be present in the tissues as a result of other causes. M. R. C. War Memorandum No. 2 (1943) states 'In some cases gas bubbles may be seen in x-ray films taken before operation and this finding may be of value in suspecting the diagnosis of anaerobic infection. It should be remembered, however, that gas may be shown apart from the existence of true gas-gangrene and its extent often has no relationship to the clinical state'. It is possible that the two cases in the present study in which no pathogenic anaerobes were isolated from the material collected may not be cases of gas-gangrene.

Table VIII is a comparative table of incidence of anærobes responsible for cases of gas-gangrene in the present series along with those of other workers :—

TABLE VIII.

Percentage incidence of the pathogenic anærobes in gas-gangrene reported by various workers.

	Weinberg and Seguin (1918).	McIntosh (1918).	Henry (1918).	MacLennan (1943).	Present series.
<i>Cl. welchii</i>	77	67.3	80	56	52
<i>Cl. oedematiens</i>	34	4	10	37	Nil.
<i>Cl. histolyticum</i>	6	20
<i>Cl. septicum</i>	13	16.3	13	19	36
<i>Cl. fallax</i>	16.5	...	6	1	...

Table VIII indicates that the incidence of pathogenic anærobes responsible for cases of gangrene varies in different countries. Thus, the incidence of *Cl. welchii* is highest in Henry's (1918) series, while a lower percentage of the incidence is reported by MacLennan (*loc. cit.*) in his study of 146 cases from the Middle East in the second world war. In the present study *Cl. welchii* accounted for 52 per cent of the cases studied. Similar variation is seen in *Cl. oedematiens*. This anærobe was not met with in any of the 25 cases studied, while its percentage varied from 4 to 37 in the cases reported by other workers mentioned in Table VIII. The same can be said of *Cl. histolyticum* and *Cl. septicum*. As the main source of anærobes is soil, the incidence of anærobes in wound infection is bound to vary according to the anærobic flora of the soil in a particular region. However, in all the cases studied, *Cl. welchii* stands out prominently as the most frequent anærobe responsible for cases of gas-gangrene either alone or in combination with other anærobes.

SUMMARY.

1. Two hundred and ten samples collected from various sources, such as fresh wounds, wounds in surgical wards or autopsy room, were submitted to the bacteriologic investigation with a view to isolating anærobic organisms. Eighty-seven strains of anærobes were isolated and identified from this material.
2. The incidence of anærobes, isolated from cases of fresh wounds, cases of tetanus, and gas-gangrene, is discussed in relation to the findings of other workers, taking into consideration the type of wounds studied.
3. *Cl. welchii* either alone or in combination with other anærobes accounted for 52 per cent of the cases of gas-gangrene investigated in the present series.

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MUTUAL INFLUENCE OF NICOTINIC ACID AND RIBOFLAVIN IN METABOLISM.

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INTRODUCTION.

WITH progress of researches on the physiological function of the different vitamins, attention has recently been directed to the investigation of their mutual influence in metabolism. Patients suffering from chronic deficiency of one particular member of B-complex are often reported to manifest the symptoms of the multiple deficiencies with regard to its other members and investigation has, therefore, been carried out in some laboratories to see whether the massive administration of one member of B-complex affects the metabolism of the others and also to see how far the state of deficiency of one member of the B vitamins affects the absorption and storage of the others.

Lehman and Nielsen (1939), Braendstrup (1940) and Salvesen (1940) observed that when the patients suffering from multiple dietary deficiencies as climacteric depression, etc., resulting from the intake of meatless, milkless and other sorts of deficient diets undergo treatment with doses of vitamin B₁ alone, the symptoms of pellagra resulting from nicotinic-acid deficiency are intensified. On the other hand Sydenstriker (1941) found that when nicotinic acid was ingested by patients suffering from pellagra, the symptoms of the deficiency in other factors were intensified. In contradiction to the work of Ferrebee and Weissman (1943) and Singher *et al.* (1944), Sure (1944, 1945) has shown that large urinary riboflavin excretion encountered in chronic thiamine deficiency in rats is not due to enhanced tissue catabolism or any change in absorption but mostly due to poor utilization of the dietary riboflavin. Klopp *et al.* (1943) failed to produce any symptom of riboflavin deficiency, either clinical or chemical, in human subjects, except some transitory rise in riboflavin excretion, after daily administration of thiamine for

as long as seventy-three days. As regards the effect of riboflavin deficiency on thiamine utilization, Sure and Ford (1942) observed that such nutritional condition does not affect the urinary excretion of thiamine.

The work discussed above indicates a possible mutual inter-relationship between thiamine and riboflavin and between thiamine and nicotinic acid, but literature so far available does not furnish any information regarding the mutual effect of riboflavin and nicotinic acid in metabolism. Since these two vitamins take essential part in various enzymatic actions, the study of the above problem may furnish some new information regarding their physiological functions, and with this possibility in view the present work on mutual inter-relationship between nicotinic acid and riboflavin has been taken up.

EXPERIMENTAL.

Twelve adult rats weighing between 168 g. and 212 g. were selected for the experiment and were given the basal diet of the composition shown in Table I. The casein used in the experiment was freed from nicotinic acid and riboflavin by repeated washings with water and alcohol. The diet prepared from such casein contained a low quantity as 0.3 μ g. of niacin and 0.02 μ g. of riboflavin per g. of food.

After preliminary depletion period of seven days on the above basal diet the urine and the faeces of the twelve rats were collected daily for a further period of three consecutive days on that diet and their nicotinic acid and riboflavin were measured. After this basal period the rats were divided into two groups, each containing six: one (group A) was then given graded doses of niacin varying from 5 μ g. to 1,000 μ g. and the other (group B) was given riboflavin in doses varying from 5 μ g. to 500 μ g. The daily collection of urine and faeces on any particular dose of the supplement was begun after a preliminary period of four days on that dose and continued for a further period of three days. When the rats of group A were given graded dose of niacin, no extra riboflavin except that available from the basal diet was given to them and thus they were made partially deficient with regard to riboflavin. Similar was the case with rats of group B which did not receive any extra nicotinic acid when graded dose of riboflavin was given to them and thus they were made partially deficient with regard to nicotinic acid. The rats were fed *ad libitum* and each consumed 8 g. to 12 g. of food per day which, however, supplied on an average 3 μ g. of nicotinic acid and 0.2 μ g. of riboflavin.

The following methods were adopted for the analysis of nicotinic acid and riboflavin:—

Nicotinic acid: By the methods of Swaminathan (1942) and Wang and Kodicek (1943).

Riboflavin: By the fluorimetric method of Slater and Morell (1946).

RESULTS AND DISCUSSION.

A. *Effect of nicotinic acid on riboflavin excretion and vice versa.*—The effect of administration of graded dose of nicotinic acid on riboflavin excretion is presented in Table I from which it will be observed that when the rats of group A

TABLE I.

Showing the effect of graded dose of nicotinic acid on riboflavin excretion.

The data represent the daily average values per rat expressed in μg . The percentage absorption and utilization of the added dose of nicotinic acid is also indicated.

Dose of nicotinic acid.	RIBOFLAVIN METABOLISM.			NICOTINIC-ACID METABOLISM.					
	Faecal output.	Urinary output.	Total output.	Faecal output.	DOSE ABSORBED.		Urinary output.	DOSE UTILIZED.	
					Total.	Per cent.		Total.	Per cent.
NIL*	4.2	6.0	10.2	16.6	36.6
5	4.0	6.3	10.3	17.0	4.6	92.0	40.8	0.4	8.7
10	5.1	6.5	11.6	17.2	9.4	94.0	43.5	2.5	26.6
20	4.8	6.1	10.9	18.6	18.0	90.0	46.8	7.8	43.3
50	4.4	7.2	11.6	20.8	45.8	91.6	49.2	33.2	72.5
500	4.3	7.6	11.9	22.4	494.2	98.8	58.7	472.1	95.5
1,000	4.5	7.5	12.0	22.8	993.8	99.38	63.1	967.3	97.3

* The rats were only given the basal diet without any additional dose of nicotinic acid and riboflavin. The basal diet was composed of casein—18 per cent, Osborne and Mendel salt mixture—4 per cent, sugar—8 per cent, starch—64 per cent and ground-nut oil—6 per cent. Vitamins A and D were given in the form of cod-liver oil in bi-weekly dose of 0.5 g. Thiamine and pyridoxine were given in daily dose of 10 μg . and 20 μg . respectively.

were kept on basal diet without any additional dose of nicotinic acid the average daily riboflavin content of urine and faeces were found to be 6.0 μ g. and 4.2 μ g. respectively. When nicotinic acid was ingested in graded doses ranging from 5 μ g. to 1,000 μ g. to the above rats, the riboflavin excretion in their faeces remained almost constant but in urine it showed slight increase. At the maximum dose of 1,000 μ g. of nicotinic acid the urinary excretion of riboflavin was found to elevate to 7.5 μ g. but this increase from 6.0 μ g. to 7.5 μ g. does not seem to be much significant in consideration of the high dose of nicotinic-acid supplement. The results show that excess administration of nicotinic acid does not affect the riboflavin excretion of the animals.

Similarly, when the rats of group B were kept on the same basal diet they excreted on an average 15.1 μ g. and 38.0 μ g. of nicotinic acid in faeces and urine respectively and these urinary and faecal excretions did not show any variation even when graded dose of riboflavin ranging from 5 μ g. to 500 μ g. was administered along with the above diet. The results, therefore, clearly indicate that massive dose of riboflavin intake does not interfere with the urinary and faecal excretions of nicotinic acid under the above dietary conditions. From the present investigation on the mutual inter-relationship between nicotinic acid and riboflavin it is evident that massive administration of one of these vitamins will not aggravate the deficiency of the other. Thus, these two vitamins are not expected to interfere with the excretions of each other even when they are present in optimum level in the diet. The fact that the total eliminations of riboflavin and nicotinic acid remain unchanged when one of them is given in excess in the diet, leads also to the supposition that massive administration of one of the above vitamins does not influence the tissue storage of the other.

It is interesting to note here that the rats fed on the basal diet supplying about 3 μ g. of nicotinic acid and 0.2 μ g. of riboflavin daily excreted on an average a large total urinary and faecal excretion as 8 μ g. to 10 μ g. of riboflavin and 50 μ g. to 55 μ g. of nicotinic acid daily. That this high elimination of riboflavin and nicotinic acid on above diet is due to microbial synthesis of these vitamins in the intestine has been observed by different workers as reviewed by Daft and Sobrell (1945) and also from recent investigation in this Laboratory (De, Datta and Roy, 1948). Mutual ineffectiveness of the added dose of riboflavin and nicotinic acid on the above basal excretions as shown in Tables I and II also suggest that microflora which synthesize riboflavin do not require nicotinic acid as their growth factor and others synthesizing nicotinic acid can grow in absence of riboflavin. Probably same species of bacteria is involved in the synthesis of both nicotinic acid and riboflavin.

B. Utilization of the added dose.—Sure (*loc. cit.*) in their experiments on thiamine-riboflavin inter-relationship have also determined the percentage absorption and utilization of the dose of riboflavin ingested to rats suffering from partial chronic deficiency on thiamine. In their experiments the percentage absorption had been calculated simply by deducting the faecal output from the dietary intake and the percentage utilization by deducting the urinary output from the quantity absorbed. From the present knowledge of the metabolism of water-soluble vitamins it is, however, known that the faecal and urinary output of any particular member of the B vitamins on any particular dose represent not only the

TABLE II.

Showing the effect of graded dose of riboflavin on the excretion of nicotinic acid.

The data represent the daily average values per rat in μg . The percentage absorption and utilization of the added dose of riboflavin is also indicated.

Dose of riboflavin.	NICOTINIC-ACID METABOLISM.			RIBOFLAVIN METABOLISM.					
	Faecal output.	Urinary output.	Total output.	Faecal output.	DOSE ABSORBED.		Urinary output.	DOSE UTILIZED.	
					Total.	Per cent.		Total.	Per cent.
Nil.*	15.1	38.0	53.1	3.6	5.3
5	17.8	36.1	54.2	3.8	4.8	96.0	6.9	3.2	66.06
25	18.1	38.7	57.1	3.8	24.8	99.2	13.0	17.1	98.90
50	16.5	37.6	54.1	5.5	48.1	96.2	21.2	32.2	66.95
100	16.5	31.7	51.2	6.4	97.2	97.2	40.10	62.4	61.19
500	16.1	36.8	52.9	8.9	491.7	98.9	191.8	308.2	62.30

* The rats were only given the basal diet without any additional dose of riboflavin and nicotinic acid.

The basal diet used here was the same as employed in the previous experiment indicated in Table I.

unabsorbed and unutilized portion of the added dose but also a certain amount which is constantly excreted even when the diet is almost free from that vitamin and these urinary and faecal excretions on a such deficient diet may be regarded as 'endogenous' in origin. Although the above basal faecal excretion is bacterial in origin as has been indicated previously, it has been designated as 'endogenous' for the reasons discussed below.

Mitchell (1929) and other investigators in their accurate determination of the digestibility of protein by animals made correction for that fraction of faecal nitrogen which is contained in substances in the animal body and which is excreted regularly even when the animals are kept on nitrogen-free diet. The nitrogen thus excreted has been termed the 'metabolic nitrogen of the faeces'—a portion of which among other substances as digestive juice, epithelial cells and mucus material, is represented by bacteria. As a rule, the bacteria cannot logically be classed under 'metabolic nitrogen' since they developed at the expense of food nitrogen as well as at the expense of that in the form of intestinal secretions, and the nitrogen of these bacteria should, therefore, be considered as food nitrogen which has been fixed in the intestine as bacterial protein and which has escaped absorption. Since this bacterial nitrogen represents a different sort of nitrogen wastage than the undigested food nitrogen, it has, however, also been classed under 'metabolic faecal nitrogen'.

In case of nicotinic acid, riboflavin and other B vitamin excretions in faeces of animals on a diet free from the above vitamins, the fact seems to be quite otherwise. In the present case there is every possibility that nicotinic acid and riboflavin excreted in faeces on the above basal diet remain fixed in the body of the bacteria the growth of which, however, depends to a certain extent on the nature of the dietary carbohydrate, but since these vitamins are synthesized by the above bacteria in the intestine from simple non-nitrogenous and nitrogenous precursors not related to these vitamins, it may be conceived that the riboflavin and nicotinic present in the faeces of the animals on deficient diet do represent neither the unabsorbed food residue nor the body vitamins wasted in the form of bacteria. Najar and Holt (1943) in their study of biosynthesis of thiamine in human subjects have shown that appreciable amount of thiamine found in the stools of the subjects neither comprised the unabsorbed food residue nor represented the excretion product of the body into the stools but was actually the synthetic product of the intestinal bacteria. In the light of the above discussion the faecal excretion of riboflavin and nicotinic acid on the above basal diet as has been recorded in the present investigation may tentatively be regarded as 'endogenous' in origin. In this connection, it is further mentioned that the above faecal excretions did not show any appreciable variation from rat to rat and this helps to substantiate the above conception of 'endogenous' faecal excretion of riboflavin and nicotinic acid. Experiments are, however, in progress to get further information on the above controversial subject.

To deduce the absolute values of absorption and utilization of the added dose of any particular vitamin the 'endogenous' faecal and urinary eliminations should be deducted from the total faecal and urinary eliminations on each dose of the vitamin under investigation. In the present investigation the above endogenous fraction has been determined by measuring the urinary and faecal output

on basal diet almost free from nicotinic acid and riboflavin, after depletion period of seven days with that diet, and the absolute values of absorption and utilization of the added dose have been calculated according to the following formula :—

Total absorbed	$D - (F_2 - F_1)$
Per cent absorption	$\frac{D - (F_2 - F_1) \times 100}{D}$
Total utilized	$D - (F_2 - F_1) - (U_2 - U_1)$
Per cent utilization	$\frac{D - (F_2 - F_1) - (U_2 - U_1) \times 100}{D - (F_2 - F_1)}$

Where D represents added dose of nicotinic acid or riboflavin.

F_2	..	total faecal output on the added dose.
F_1	..	'endogenous' faecal output on basal diet.
U_2	..	total urinary output on added dose.
U_1	..	'endogenous' urinary output on basal diet.

Calculating in the above manner it has been observed that at all levels of intake the percentage absorption of the added dose of nicotinic acid and riboflavin remained constant in the region of 90 to 100 (Tables I and II). When their utilization is considered these two vitamins, however, seem to behave in different manners. In case of riboflavin the percentage utilization of the added dose at all levels of intake from 5 μ g. to 500 μ g. was found to lie almost unchanged in the range of 60 to 70, but in case of nicotinic acid the above value showed tendency to increase with the increase of the dose. When this acid was ingested at the level of 5 μ g. per day (Table II) almost all the ingested dose was excreted through the urine and only a small per cent as 8.7 of the added dose was found to be utilized in the body. On the contrary when the dose was raised to 1,000 μ g. per day as large an amount as 97.8 per cent of the added dose was utilized in the body.

The results of Sure (*loc. cit.*) in which this endogenous source has been totally discounted do not seem to represent the 'absolute' values of absorption and utilization of the added dose of riboflavin.

In a previous communication from this Laboratory (De and Banerjee, 1948) it has been shown that human subjects possess a limited capacity to excrete nicotinic acid through the urine which, however, cannot be raised even when higher dose is administered. So with regard to the utilization of nicotinic acid rats and human subjects behave in quite different manners.

SUMMARY.

1. Rats kept on diet having negligible amount as 0.3 μ g. nicotinic acid and 0.02 μ g. riboflavin per gramme of food cast out on an average a total urinary and faecal elimination of 50 μ g. to 55 μ g. nicotinic acid and 8 μ g. to 10 μ g. riboflavin per day.

2. The above excretion of nicotinic acid was not affected even when graded dose of riboflavin varying from 5 μ g. to 500 μ g. was ingested and likewise the riboflavin excretion remained unchanged when nicotinic acid was administered in daily dose varying from 5 μ g. to 1,000 μ g.

3. It is concluded that massive administration of one of the above two vitamins does not influence the absorption and the storage of the other.

4. Modified method has been described for calculating the percentage absorption and utilization of the added dose of B vitamins by taking into consideration the 'endogenous' urinary and faecal output derived from the microbial synthesis in the intestine of rats kept on diet free from the vitamin under investigation.

5. The percentage absorption of the added dose of nicotinic acid and riboflavin at all levels of intake was found to remain constant in the region of 90 to 100.

6. The percentage utilization of riboflavin was found to remain unchanged in the range of 60 to 70 at all levels of intake, whereas that of nicotinic acid increased from 8.7 to 97.8 with the increase of the dose from 5 μ g. to 1,000 μ g.

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COPPER AND MANGANESE METABOLISM WITH TYPICAL INDIAN DIETARIES AND ASSESSMENT OF THEIR REQUIREMENT FOR INDIAN ADULT.

BY

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INTRODUCTION.

COPPER and MANGANESE form the normal constituents of all tissues of animal and plant kingdom but their importance in nutrition has been realized only in recent years. The literature regarding the human requirement of these nutritionally essential elements is meagre.

Schoular (1936) showed that the children of ages between 3 and 6 years required 0.053 mg. to 0.085 mg. of copper per kilogram body-weight. Daniels and Wright (1934), by fifteen balance studies on eight children ranging from 4 to 6 years, have shown that they required 0.100 mg. of copper per kilogram body-weight. The investigations of Chou and Adolph (1935) carried out on adult men and of Leverton and Binkley (1944) on adult women have shown that copper requirement should range between 2.0 mg. and 2.5 mg. per day. The unpublished data of Basu and Gupta (1946) from this Laboratory have indicated the value of the copper requirement in the range of 2.3 mg. per day.

Everson and Daniels (1934) on studying twelve manganese-balance experiments with seven children of 4 to 6 years of age at three levels of ingestion suggested that the diet for children should contain between 0.20 mg. and 0.30 mg. of manganese per kilogram body-weight. Basu and Malaker (1940) working with normal adults in this Laboratory found the manganese requirement to be 4.6 mg. per day.

The present work has been taken up to investigate the copper and manganese requirement of normal Indian adult by statistical analysis of the data of output at different levels of intake and to study how far the Indian rice and wheat diets can satisfy these requirements.

EXPERIMENTAL.

Thirty balance studies of copper and twenty-seven of manganese metabolism have been carried out on nine male adults. The experimental diets, subjects, procedure and the technique of collection of urine and faeces were the same as that reported in our previous paper (De and Basu, 1949).

Copper was estimated by the method of Callan and Henderson (1929) as modified by McFarlane (1932) and Hoar (1937) by the use of the reagent di-ethyl-di-thiocarbamate and manganese by the method of Skinner and Peterson (1930). Since only minute traces of manganese were found in the urine, only the faecal output was estimated in case of manganese metabolism. All possible precautions were adopted to avoid any contamination. Aluminium utensils were used for cooking the diets and the analyses were carried out in a specially prepared glass room.

RESULTS AND DISCUSSION.

Metabolic studies with rice and wheat diets.

Copper.—The results presented in Table I show that the copper content of the rice diets (both vegetarian and non-vegetarian) varies from 2.38 mg. to 6.28 mg. with the mean value of 4.53 mg. On wheat diet the intake ranged from 4.13 mg. to 8.64 mg. with the mean value of 5.83 mg.

At all levels of intake on both rice and wheat diets the balance of copper remained positive and increased steadily with the increase in the level of intake. The balance of copper on rice diet ranged from + 0.26 mg. to + 3.62 mg. with the mean value of + 1.51 mg. and on wheat diet ranged from + 1.09 mg. to + 4.35 mg. with the mean value of + 2.44 mg.

Manganese.—In case of rice diets the intakes of manganese (Table II) ranged from 7.28 mg. to 12.43 mg. with the mean value of 9.81 mg. and in case of wheat diet, with the exception of one having the dietary content as low as 6.42 mg., the intake ranged from 7.14 mg. to 13.81 mg. with the mean value of 9.61 mg. The average balance on rice and wheat diets were found to be + 3.21 mg. and + 3.28 mg. respectively.

The above results show that both the rice and wheat diets can keep the normal subjects in high positive balances of copper and manganese.

Metabolic studies with sago diet.

Four metabolic experiments on sago diet containing 550 g. to 600 g. sago, 200 g. sugar and 50 g. ghee (butter-fat) have been carried out on two normal individuals and the metabolic results have been presented in Table III. The data shows that this diet can supply just an adequate amount of copper to keep the normal adult in positive balance. On an average intake of 2.83 mg. of copper the average balance was found to be on the positive side of + 0.74 mg. As regards manganese the subjects on this diet maintain negative balance. On an average intake of 0.71 mg. the mean output was found to be 1.76 mg. and the subjects thus showed a deficit of - 1.05 mg. of manganese per day.

TABLE I.

Copper metabolism with rice and wheat diets.

The figures indicate the daily averages in mg. for intakes, outputs and balances over six or nine days.

Experimental diet.	Experimental subject.	Dietary intake of Cu.	Urinary output of Cu.	Faecal output of Cu.	Total output of Cu.	Balance per day.
Vegetarian rice diet	P. C. D. ...	2.73	0.41	1.56	1.97	+0.76
	S. G. P. ...	3.85	0.28	2.65	2.93	+0.92
	P. C. G. ...	3.88	0.39	2.18	2.57	+1.31
	G. C. D. ...	3.91	0.52	2.70	3.22	+0.69
	P. C. D. ...	4.33	0.38	2.88	3.26	+1.07
Rice-fish diet	G. C. D. ...	2.38	0.29	1.83	2.12	+0.26
	P. C. D. ...	2.98	0.32	2.15	2.47	+0.51
	G. C. N. ...	3.05	0.37	1.28	1.65	+1.40
	P. C. G. ...	4.06	0.39	2.16	2.55	+1.51
	G. C. N. ...	4.08	0.34	1.89	2.23	+1.85
	K. R. G. ...	4.24	0.21	3.16	3.37	+0.87
	K. R. G. ...	4.52	0.60	2.20	2.80	+1.72
	K. R. G. ...	4.62	0.71	2.69	3.40	+1.22
	G. C. D. ...	4.93	0.35	3.01	3.36	+1.62
	G. C. D. ...	5.12	0.42	3.18	3.60	+1.52
	G. C. N. ...	5.24	0.40	3.28	3.68	+1.56
	G. C. N. ...	5.31	0.31	2.71	3.02	+2.29
	H. P. D. ...	5.61	0.34	3.00	3.34	+2.27
	H. P. D. ...	6.13	0.56	3.52	4.08	+2.05
	H. P. D. ...	6.15	0.38	2.15	2.53	+3.62
Average	G. C. N. ...	6.18	0.58	3.14	3.72	+2.46
	P. C. D. ...	6.28	0.41	4.15	4.56	+1.72

Whole-wheat diet	K. R. G. ...	4.13	0.53	2.51	3.04	+1.09
	G. C. N. ...	4.28	0.34	1.82	2.16	+2.12
	G. C. N. ...	5.26	0.52	2.27	2.79	+2.47
	G. C. N. ...	5.28	0.49	2.28	2.77	+2.51
	G. C. N. ...	5.44	0.51	3.58	4.09	+1.35
	P. C. G. ...	6.45	0.32	4.15	4.47	+1.98
	G. C. D. ...	7.18	0.31	3.20	3.51	+3.67
	P. C. D. ...	8.64	0.38	3.91	4.29	+4.35
Average

Average		5.83	0.42	2.97	3.39	+2.44

TABLE II.

Manganese metabolism with typical rice and wheat diets.

The figures indicate the daily averages in mg. for intakes, outputs and balances over six or nine days.

Experimental diet.	Experimental subject.	Dietary intake of Mn.	Fæcal output of Mn.	Balance per day.
Vegetarian rice diet ...	G. C. D. ...	7.58	5.45	+2.13
	G. C. D. ...	7.59	6.45	+1.14
	G. C. D. ...	8.47	5.32	+3.15
	P. C. G. ...	10.79	6.61	+4.18
Rice-fish diet ...	H. P. D. ...	7.28	5.05	+2.23
	P. C. D. ...	8.07	4.77	+3.30
	S. G. P. ...	8.89	5.97	+2.92
	G. C. N. ...	9.41	6.42	+2.99
	G. C. D. ...	9.48	5.86	+3.62
	K. R. G. ...	9.71	6.48	+3.23
	K. R. G. ...	9.81	6.38	+3.43
	G. C. D. ...	9.88	5.72	+4.16
	G. C. N. ...	9.81	6.70	+3.11
	K. R. G. ...	10.12	7.58	+2.54
	G. C. N. ...	10.18	6.14	+4.04
	G. C. D. ...	10.94	8.60	+2.34
	G. C. N. ...	10.98	6.54	+4.44
	H. P. D. ...	11.24	6.89	+4.35
	G. C. N. ...	11.28	8.34	+2.94
	H. P. D. ...	12.18	8.74	+3.44
	P. C. D. ...	12.43	8.61	+3.82
Average	9.81	6.60	+3.21
Whole-wheat diet ...	G. C. N. ...	6.42	3.58	+2.84
	G. C. N. ...	7.14	4.49	+2.65
	K. R. G. ...	7.28	5.56	+1.72
	G. C. N. ...	7.38	5.14	+2.24
	P. C. D. ...	12.14	7.63	+4.51
	G. C. N. ...	13.14	8.28	+4.86
	P. C. D. ...	13.81	9.66	+4.15
Average	9.61	6.33	+3.28

TABLE III.

Copper and manganese metabolism with sago diet.

The figures indicate the daily averages in mg. for intakes, outputs and balances over six days.

Diet.	Experimental subject.	COPPER METABOLISM.					MANGANESE METABOLISM.		
		Dietary intake.	Urinary output.	Faecal output.	Total output.	Balance.	Dietary intake.	Faecal output.	Balance.
Sago	G. C. N. ...	2.91	0.20	2.14	2.34	+ 0.57	0.74	1.16	- 0.42
		3.37	0.35	2.08	2.43	+ 0.94	0.70	2.09	- 1.39
	G. C. D. ...	2.41	0.54	1.12	1.66	+ 0.75	0.69	1.38	- 0.69
		2.63	0.45	1.48	1.93	+ 0.70	0.71	2.41	- 1.70
Average	2.83	0.38	1.71	2.09	+ 0.74	0.71	1.76	- 1.05

ASSESSMENT OF MINIMUM REQUIREMENTS OF COPPER AND MANGANESE.

To evaluate the minimum requirement of a particular element for proper nutrition it is necessary to have some data of intake which almost balance the output. In the present investigation, only in case of sago diet (Table III) a few data of intakes are available at which the balances of copper and manganese become almost zero. These intakes at low levels give only a rough estimate of the minimum requirement for copper and manganese. In order to determine these values more accurately, the data of intakes and balances presented in Tables I, II and III have all, therefore, been treated statistically in a way similar to that adopted by Leitch (1937) and Leitch and Duckworth (1937) in their assessment of the minimum requirements of protein and calcium.

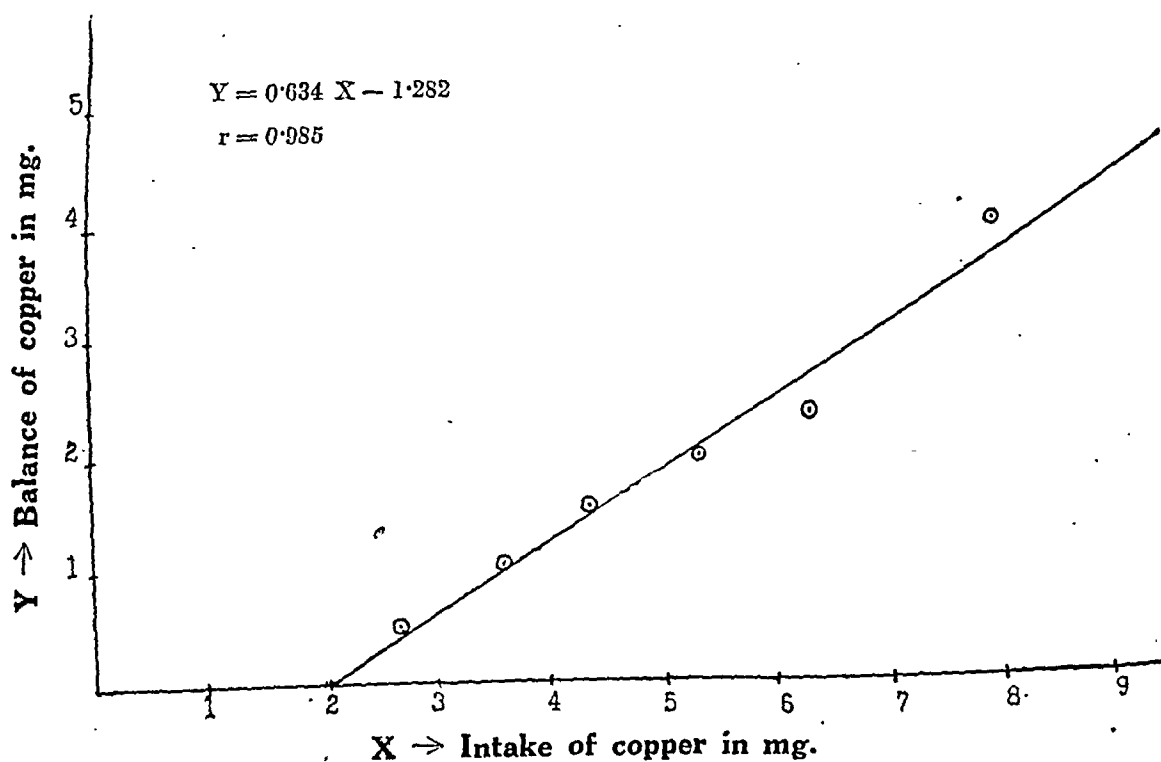
The intakes have been grouped according to different ranges (Table IV) and the mean values of balances corresponding to the mean values of intakes at different range-groups have been fitted up in a regression line—each separately for copper and manganese (*see* Graphs 1 and 2).

TABLE IV.

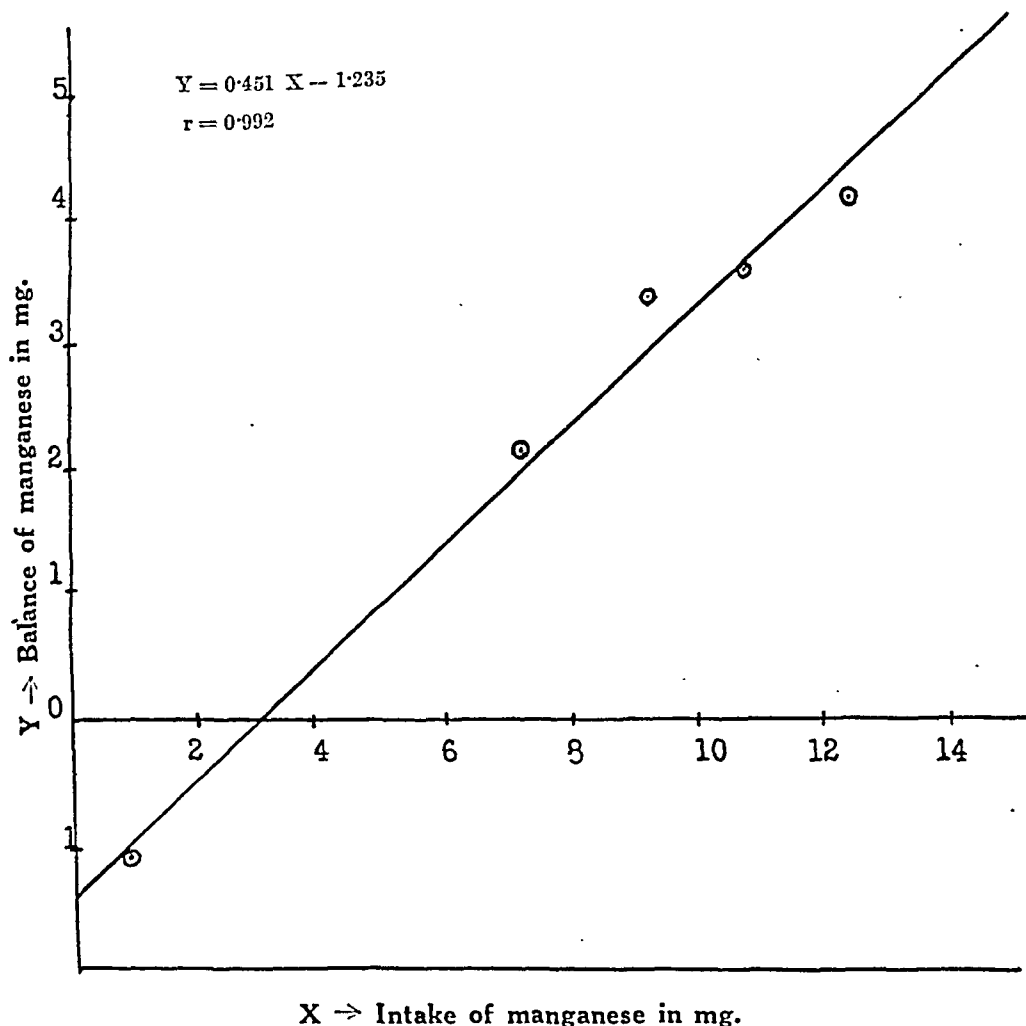
Showing the mean values of balances corresponding to mean values of intakes at different ranges.

The figures are expressed in mg. per day.

COPPER METABOLISM.			MANGANESE METABOLISM.		
Range of intake.	Mean intake.	Mean balance.	Range of intake.	Mean intake.	Mean balance.
2.00 to 2.99	2.69	+0.51	0.00 to 0.99	0.71	-1.05
3.00 to 3.99	3.61	+1.05	6.00 to 7.99	7.25	+2.13
4.00 to 4.99	4.33	+1.56	8.00 to 9.99	9.28	+3.32
5.00 to 5.99	5.33	+1.99	10.00 to 11.99	10.79	+3.55
6.00 to 6.99	6.24	+2.34	12.00 to 13.99	12.47	+4.15
7.00 to 8.99	7.91	+4.01



GRAPH I.—Regression line for copper requirement.



GRAPH 2.—Regression line for manganese requirement.

The values of 'r' were found to be 0.985 and 0.992 for copper and manganese respectively and were tested to be highly significant. $Y = 0.634 X - 1.282$ and $0.451 X - 1.235$ represent the relationship between the balances and the intakes of copper and manganese respectively. At zero values of Y, X becomes 2.02 and 2.74 for copper and manganese respectively expressed in mg. and these values of X tentatively represent the minimum requirements of copper and manganese for normal Indian adult. Further data of balances of these elements at different levels of intakes are accumulating in this Laboratory to obtain the above requirements conclusively.

The minimum copper requirement of 2.02 mg. as deduced in the above way corroborates with the minimum limit as suggested by Chou and Adolph (*loc. cit.*) and Leverton and Binkley (*loc. cit.*) in their studies on adult men and women.

The higher value of manganese requirement of 4.6 mg. as deduced by Basu and Malaker (*loc. cit.*) is probably due to insufficient number of balance observations from which the above value of the minimum requirement has been calculated.

Effect of fat on the metabolism of copper and manganese.

It is observed from the results presented in Tables I, II and III that the balances of copper and manganese progressed steadily with the increase in the level of intakes. Although the type of the fat used in rice, wheat and sago diets was different—mustard oil in rice diet and ghee (butter-fat) in wheat and sago diets—the higher values of intakes and balances due to wheat diet and lower value due to rice diet have fitted in the same regression line in both the cases of copper and manganese. It is, therefore, evident that the nature of the fat does not influence the metabolism and requirements of copper and manganese.

SUMMARY.

1. Metabolic studies of copper and manganese on seven normal male adults with rice and wheat diets have shown that the typical rice and wheat diets of India contain sufficient amount of copper and manganese to keep the normal individual in positive balance with these elements.

2. By fitting up the regression line of balances on intakes, the minimum requirements of copper and manganese have been calculated to be 2.02 mg. and 2.74 mg. per day respectively.

3. The type of the fat used in the preparation of the diets does not affect the metabolism and requirements of copper and manganese.

Thanks are due to Dr. K. P. Basu for his kind encouragement in the work.

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DETERMINATION OF VITAMIN A WITH THE LUMETRON PHOTO-ELECTRIC COLORIMETER.

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INTRODUCTION.

THE assay of vitamin A is carried out by biological, chemical and physical methods. The curative rat-growth assay (biological) is one of the most difficult bio-assays requiring skilful attention and meticulous care in the actual test, calculations and interpretations of data. It is tedious, time-consuming and expensive, and the results are liable to variation as in the case of most bio-assay procedures. It is not applicable in routine assays, but must be used occasionally in checking the results of standardization by chemical and physical methods.

The most widely used chemical method is the Carr-Price reaction, where the blue colour formed when vitamin A is treated with antimony trichloride is measured photo-electrically. The chief disadvantages of this method are that the blue colour fades rapidly, the reagent is corrosive and is extremely sensitive to moisture, and often non-typical colours are produced.

The physical method most commonly employed is spectrophotometric, which is based on the measurement of light absorption of the vitamin in solution. The maximum absorption band is $328\text{ m}\mu$ ($324\text{ m}\mu$ to $328\text{ m}\mu$) and it is proportional to the concentration of the vitamin. The solvents used for the absorption is either ethyl alcohol or iso-propyl alcohol or cyclohexane. Although the specificity of this method is not as high as in the chemical method due to possible non-specific absorption, the precision and reproducibility are higher in the spectrophotometric method.

USE OF LUMETRON PHOTO-ELECTRIC COLORIMETER.

The intensity of absorption can be measured either by means of ultra-violet spectrograph or by the use of a photo-electric spectrophotometer. The use of the latter instrument for the estimation of vitamin A is of recent origin, while the spectrograph has been used for this purpose for quite a long time. Beckman photo-electric quartz spectrophotometer model D.U. (Cary and Beckman, 1941) has been adopted as the standard instrument for vitamin A analysis. Lumetron photo-electric colorimeter and fluorescence meter 402 E.F., manufactured by Photovolt Corporation, New York, can also be used for this purpose. The optical system is represented in the following Diagram:—

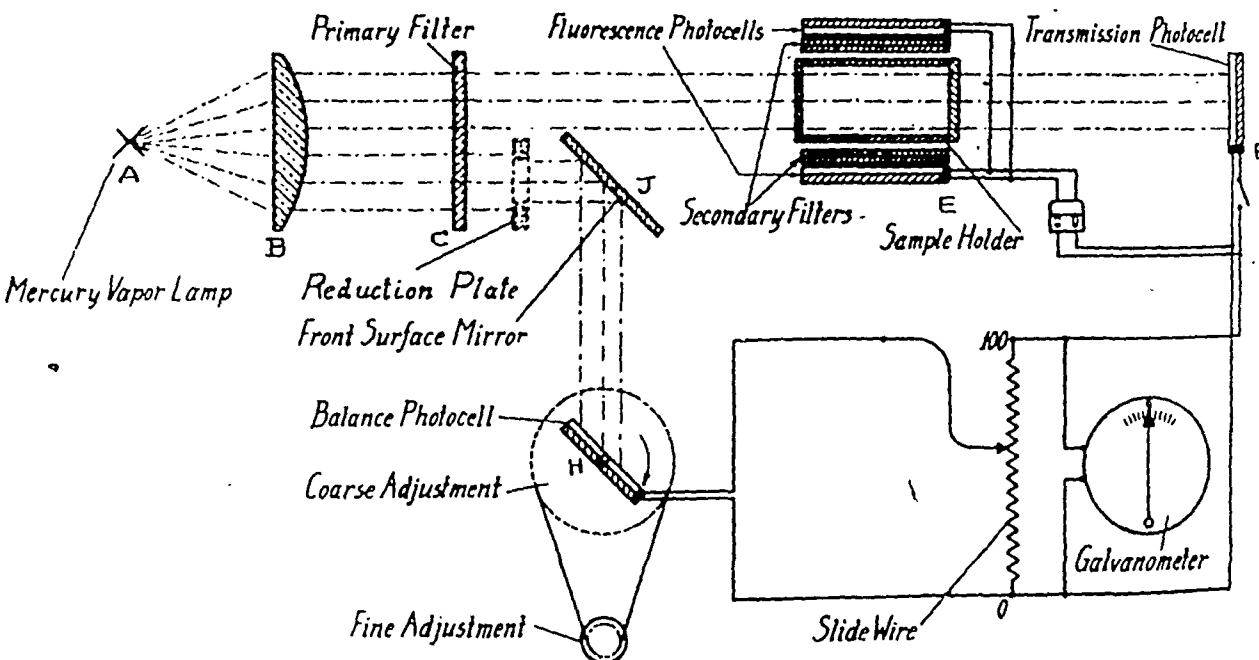


DIAGRAM.—Showing the optical system and electrical circuit of the Lumetron photo-electric fluorescence meter, Model 402 E.F.

Light from ultra-violet lamp A passes through the optical system B, filter C and is split into two parts. One part of the beam passes through a special liquid-filter D and the sample holder E and strikes the measuring photo-cell F. The other part of the beam is deflected by the mirror J and acts upon the balance photo-cell H which is mounted so that it can be turned through an angle of 90° . The measuring photo-cell and the balance photo-cell are connected in a bridge-circuit with a slide wire potentiometer and a galvanometer as zero-indicator. The I_0 adjustment to balance the two photo-cells with solvent only in the sample holder is made by rotating the standard photo-cell H. Radiation of a mercury-vapour lamp passes through the combined glass-filter and the liquid-filter which transmits only the characteristic absorption band of vitamin A. This beam then passes through the sample under test and strikes a barrier layer photo-cell. The current output of this photo-cell serves as a measure of the absorption of the sample and, therefore, as a measure of its vitamin A potency.

The instrument must first be calibrated by means of a vitamin A standard. A sample of pure vitamin A with a potency of 1,500,000 I.U. obtained from the British Drug Houses, Ltd., London was used for this purpose. The standard is diluted with iso-propyl alcohol and a series of dilutions of known concentrations are prepared. First the absorption cell is filled with iso-propyl alcohol, the slide wire dial set to 100 and the circuit is balanced by means of the balance-cell control. Then the other absorption cell is filled with the diluted standard and inserted into the instrument. The circuit is re-balanced with the slide wire and the slide wire dial setting is read. The ultra-violet transmission of these known concentrations is read on the instrument and the logarithm of readings obtained are plotted against concentration. This leads to a straight calibration line which intersects the transmission axis at the 100 point. The concentration of unknown samples can then be found easily from this graph once their ultra-violet transmission has been determined by measurement on the instrument.

EXPERIMENTAL.

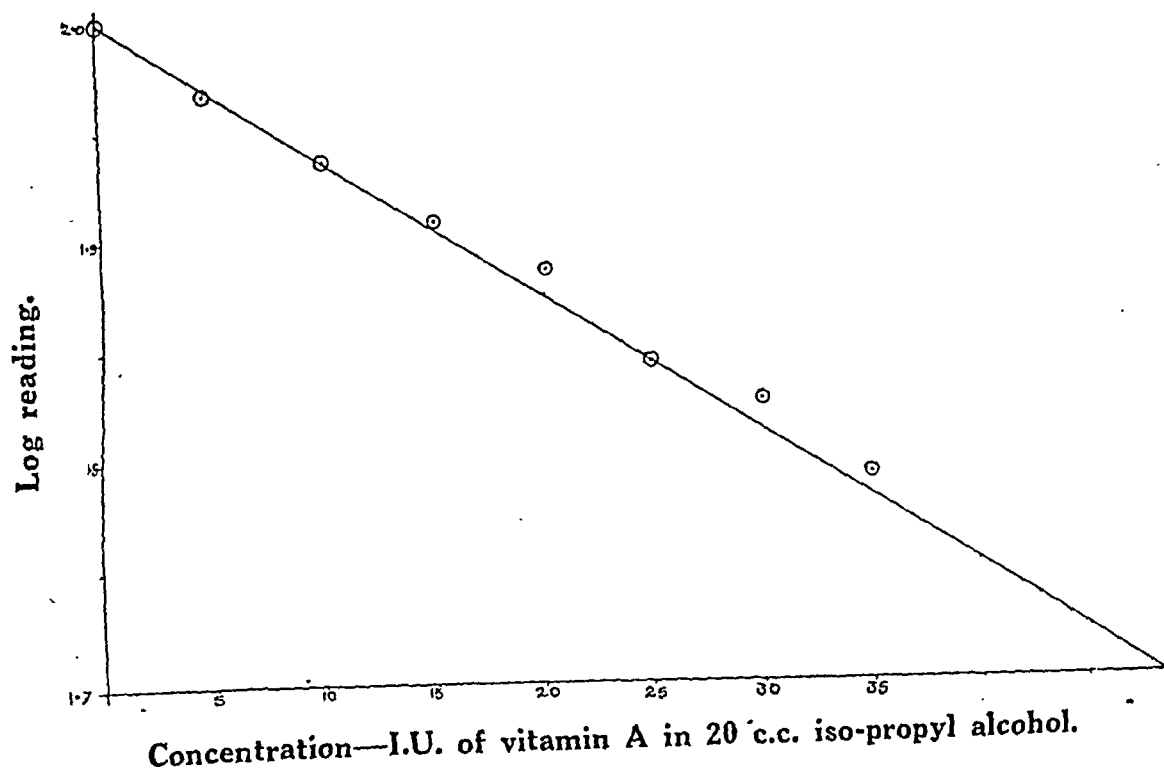
A quantity of 0.0050 g. of vitamin A (B. D. H.), the potency of which was known to be 1,500,000 I.U. per gramme, was dissolved in 15 c.c. iso-propyl alcohol. Each cubic centimetre of this solution has, therefore, a potency of 500 I.U. Half c.c. of this solution was diluted to 25 c.c. with iso-propyl alcohol, so that the resulting solution has a potency of 10 units per c.c. From this stock solution, seven solutions of different concentrations of vitamin A were prepared by diluting with iso-propyl alcohol and their ultra-violet transmissions were determined. The results are given in Table I and the calibration line drawn on the basis of these measurements is given in the Graph:—

TABLE I.

	Transmis- sion.	Log trans- mission.	Calculated conversion factor.
(iso-propyl alcohol) blank	100	2	...
(1) 0.5 c.c. of stock solution in 20 c.c. iso-propyl alcohol (5 units vitamin A in 20 c.c.).	92.75	1.9673	1,532
(2) 1.0 c.c. of stock solution in 20 c.c. iso-propyl alcohol (10 units vitamin A in 20 c.c.).	86.5	1.9370	1,590
(3) 1.5 c.c. of stock solution in 20 c.c. iso-propyl alcohol (15 units vitamin A in 20 c.c.).	82.0	1.9138	1,744
(4) 2.0 c.c. of stock solution in 20 c.c. iso-propyl alcohol (20 units vitamin A in 20 c.c.).	76.5	1.8837	1,714
(5) 2.5 c.c. of stock solution in 20 c.c. iso-propyl alcohol (25 units vitamin A in 20 c.c.).	70.25	1.8466	1,633
(6) 3.0 c.c. of stock solution in 20 c.c. iso-propyl alcohol (30 units vitamin A in 20 c.c.).	67	1.8261	1,728
(7) 3.5 c.c. of stock solution in 20 c.c. iso-propyl alcohol (35 units vitamin A in 20 c.c.).	62.25	1.7941	1,702

GRAPH.

Calibration curve.



The conversion factor has been calculated for each concentration by the application of the formula:—

$$\frac{\text{Units of vitamin A per gramme of the sample} \times c \times d}{2 - \log T} = \text{Conversion factor.}$$

T = Reading indicated by Lumetron (transmission).
 d = Thickness of the cell in cm.
 c = Concentration in per cent.

The average conversion factor is 1,663 which agrees well with the factor at present employed in all spectrographic determinations of vitamin A.

The next step was to test the applicability of this method for the determination of vitamin A in fish-liver oils, in vitaminized oils, in vitamin capsules, etc. Although a combination of liquid and glass-filters are used to cut off practically all radiations of wave lengths above 330 μ , it is not certain that oils definitely known not to contain vitamin A will not show any significant absorption. Arachis oil and olive oil, which are known to be devoid of vitamin A, showed significant absorptions when dissolved in iso-propyl alcohol and tested in the instrument. This was a serious objection for the application of this method for vitamin A determinations directly in oils. The unsaponifiable matters of these oils also showed similar absorption. The absorptions observed in various concentrations of the different oils are given in Table II.

TABLE II.

Percentage absorption in various concentrations of oil.

Concentration :—				0.1	0.05	0.03	0.015
Arachis oil	{ Direct	15.5	8.0	1.5	Nil.
	{ Unsaponifiable	14.0	7.0	1.0	Nil.
Olive oil	{ Direct	16.0	8.5	1.5	Nil.
	{ Unsaponifiable	15.0	7.5	1.0	Nil.

The absorption is progressively reduced with decrease in concentration until finally in a concentration of 0.015 per cent in iso-propyl alcohol, there is no absorption at all and in a concentration of 0.03 per cent, there is negligible absorption. It is evident from the results in Table I that the least quantity of vitamin A measurable with a fair amount of accuracy by this method is 10 units in 20 c.c. of iso-propyl alcohol, the transmission being 86.5 per cent. It follows, therefore, that oils with a vitamin A potency of more than 3,300 I.U. per gramme can be submitted to this method of vitamin A determination. Oils of this potency will have in a concentration of 0.015 per cent in iso-propyl alcohol 49.5 I.U. of vitamin A in 100 c.c. or 9.9 units in 20 c.c. which is approximately the lower limit for accurate measurement by this method.

In order to test the accuracy of the method as determined directly on oils, a known amount of standard vitamin A was added to a definite amount of arachis oil, so that the resulting preparation had a potency of about 3,000 I.U. An aliquot part of this mixed oil was saponified and the unsaponifiable matter dissolved in a known amount of iso-propyl alcohol. The transmissions of the solutions of the mixed oil direct and that of its unsaponifiable matter in iso-propyl alcohol were determined and the results are given in Table III :—

TABLE III.

	Transmission.	Observed potency as obtained by reference to graph.	Real potency.
1 c.c. of (A) diluted to 2 c.c. in iso-propyl alcohol	87.50	9.5	10
2 c.c. of (A)	74.50	20.5	20
1 c.c. of (B)	87.25	9.5	10
2 c.c. of (B)	76.00	19.0	20

0.0046 g. standard vitamin A was thoroughly mixed with 2.1022 g. of arachis oil. One g. of this preparation has, therefore, a potency of 3,276 I.U.

0.05 g. of this mixed oil (163.8 I.U. of vitamin A) was dissolved in 16.4 c.c. of iso-propyl alcohol.

One c.c. of this solution has a potency of 10 I.U.

0.1 g. of the mixed oil was saponified by the B. P. method and the final solution was made up in 32.75 c.c. of iso-propyl alcohol instead of cyclohexane. Hence, 1 c.c. of this solution contains 10 I.U. of vitamin A (B).

There is very good agreement between the actual and the observed potency. There is also no difference between the results obtained by measurement directly on the oil and on the unsaponifiable matter. These results clearly demonstrate the utility and the accuracy of the method for the determination of vitamin A in oils having a potency of more than 3,000 I.U.

The method was employed for the determination of vitamin A in oils with a potency of about 1,000 I.U. per gramme. The results are given in Table IV :—

TABLE IV.

	Transmission.	Observed potency as obtained by reference to graph.	Actual potency.
1 c.c. of (C) made up to 20 c.c. with iso-propyl alcohol.	78.5	17.0	10
2 c.c. of (C) 	61.0	35.0	20
1 c.c. of (D) 	79.5	16.5	10
2 c.c. of (D) 	62.5	33.0	20

0.0036 g. of standard vitamin A was mixed with 5.4 g. of arachis oil and the resulting preparation had a potency of 1,000 I.U. per gramme.

0.1 g. of this mixed oil was dissolved in 10 c.c. iso-propyl alcohol and 1 c.c. of this solution contains 10 I.U. (C).

0.1 g. of the mixed oil was saponified and the unsaponifiable matter was dissolved in 10 c.c. iso-propyl alcohol and similarly 1 c.c. of this solution also contains 10 I.U. ... (D).

The values obtained by experiment are considerably more than the actual potencies, which is presumably due to non-specific absorption.

Oils with a potency of 1,000 I.U. of vitamin A when tested by the instrument either directly or after saponification do not give satisfactory results. The usefulness of this instrument is, therefore, limited to vitamin A preparations with potencies of more than 2,500 to 3,000 I.U. per gramme.

The relationship between the Carr-Price blue value and the I.U. as determined with the Lumetron was investigated. The blue values of some samples of fish-liver oils, vitaminized oils, and capsules were determined by the B. P. (1932) method and the transmissions on the same samples after suitable dilution with iso-propyl alcohol were measured with the Lumetron and the corresponding potencies were obtained by reference to the calibration curve. The results are given in Table V.

TABLE V.

Relationship between blue value and potency.

		C.-P. blue value.	Potency determined by the Lumetron.	Factors for conversion of blue value to I.U.
Fish-liver oils ...	{ 1	50	3,000	60
	{ 2	80	4,500	56
	{ 3	50	2,600	52
Vitaminized oils ...	{ 4	140	8,500	61
	{ 5	200	12,200	61
	{ 6	35	2,000	57
Capsules ...	{ 7	72	4,000	55
	{ 8	100	6,000	60
	{ 9	100	5,000	50

Average factor for conversion of blue units to I.U. = 57.

SUMMARY AND CONCLUSIONS.

It appears from all the foregoing data that the Lumetron with the combined glass and special liquid-filter can be employed for all routine determinations of vitamin A in fish-liver oils, vitaminized oils, tablets and capsules having a potency of more than 2,500 I.U. This instrument will be particularly useful for studying the deterioration on storage of vitamin A in oils, tablets and capsules. Work in this direction is in progress.

1. The application of Lumetron 402 E.F. with the combined glass and special liquid-filter for the determination of vitamin A has been investigated.

2. The relationship between logarithm of transmission and potency of vitamin A has been determined by employing vitamin A of a potency of 1,500,000 units per gramme supplied by British Drug Houses, Ltd., London. The calibration curve is a straight line in the range of concentrations tested.

3. The non-specific absorptions of various concentrations of arachis and olive oils in iso-propyl alcohol, not containing any vitamin A, have been determined. It is found that these oils in concentrations of 0.015 per cent do not show any absorption, while in concentrations above 0.03 per cent, there is a gradual increase in non-specific absorption interfering with the estimation of vitamin A.

4. Oils not containing vitamin A have been mixed with standard vitamin A to obtain a concentration of 3,300 and 1,500 units per gramme and these

preparations have been tested with the instrument both directly as well as after saponification. Preparations with a potency of more than 3,000 units give very accurate results, while for those containing 1,500 units, the values obtained are considerably higher than the actual potencies presumably due to the non-specific absorption of the oil being added into the specific absorption of vitamin A.

5. Carr-Price blue values on fish-liver oils, vitaminized oils and capsules were determined and compared with the potencies as determined by the Lumetron. The average factor for the conversion of blue values to I.U. has been determined. As the results obtained show the inconsistencies of the blue values, the conversion factor cannot be ascertained with any degree of accuracy or certainty. Figure 57, however, can be used as a rough guide for all practical purposes.

6. It is concluded that the Lumetron 402 E.F., with the combined glass- and special liquid-filter supplied for vitamin A, can be safely employed for the accurate determination of vitamin A in preparations with a potency of more than 3,000 units. This instrument will be particularly useful for the study of deterioration of vitamin A in oils, capsules and tablets.

Thanks are due to Dr. B. Mukerji, Director of the Laboratory, for his interest and suggestions in this study.

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SUPPLEMENTARY VALUE OF LUCERNE TO THE POOR SOUTH INDIAN RICE DIET.

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THE low nutritive value of the South Indian rice diet has been the subject of extensive earlier investigations (Aykroyd and Krishnan, 1937, 1937*a*, 1937*b*). It is one of the poorest diets of the world, especially when it is strictly vegetarian and includes little or no milk, as is the case with the poor classes. It is essentially a carbohydrate diet with gross deficiencies in protein, minerals and vitamins. The more obvious methods of supplementing such a diet to improve the nutritive value are precluded by either economic or religious considerations.

The high nutritive value of leafy vegetables has been described very fittingly by McCollum *et al.* (1939), '... the leaf of a plant is a complete food, whereas none of the storage organs of the plants, seeds, tubers, roots or fruits, enjoy this distinction. . . The leaf is the site of synthesis of proteins, carbohydrates and fats and is rich in actively functioning cells. These cells contain everything which is necessary for the metabolic processes.' Having found a pleasant taste in a sample of a leaf flour made from artificially dried young oat leaves, they continue to write, 'It would seem that there may be a future in human nutrition for such leaf flours. . . Certainly such leaf flours have very high nutritive values as respects all the essentials of an adequate diet.'

More recently, the existence of growth-promoting factors in grass juice has come to be recognized (Kohler *et al.*, 1936, 1938; Mannering *et al.*, 1943). Valuable work has been done by Basu and Ghosh (1943, 1943*a*) in demonstrating the high nutritive value of different leafy foods. If it can be demonstrated that such

cheap and abundantly available materials have a high supplementary value, there will then be scope for a practical approach to the food problem not only in India, but also in other parts of the world which are similarly placed.

Lucerne, a rich source of proteins, minerals and vitamins, is already being grown quite extensively both in this country and elsewhere. Under favourable conditions, an yield of 60 tons or more per acre can be expected. One cutting for every three or four weeks is possible for a number of years. The carotene and vitamin K contents of lucerne are higher than those in any other leafy vegetables (Heupke and Schöller, 1943). It contains twice the amount of iron present in spinach and four times the vitamin C present in citrus fruit (Levy and Fox, 1935). It is also rich in calcium, phosphorus and vitamin E. McCollum *et al.* (1917) showed the supplementary value of lucerne to cereals. Levy and Fox (*loc. cit.*), Marston *et al.* (1943) and Heupke and Schöller (1942) have suggested the use of lucerne as human food.

EXPERIMENTAL.

In the present study, the supplementary value of dry lucerne (tender leaves dried in a current of air at 65°C. and then powdered to pass 30-mesh sieve) when it partly replaces rice in the diet has been investigated. The composition of the diet was the one adopted by the Vanaspati Research Committee of the Food Ministry and consists mainly of the following:—

Polished rice 78.5 per cent; tur dhal (*Cajanus indicus*) 5 per cent; salt (NaCl) 0.3 per cent; non-leafy vegetables 8.2 per cent; leafy vegetables 2.1 per cent; whole-milk powder 0.9 per cent; and crude ground-nut oil 5 per cent. The milk powder was not mixed with the diet but dissolved in water and fed separately in cups.

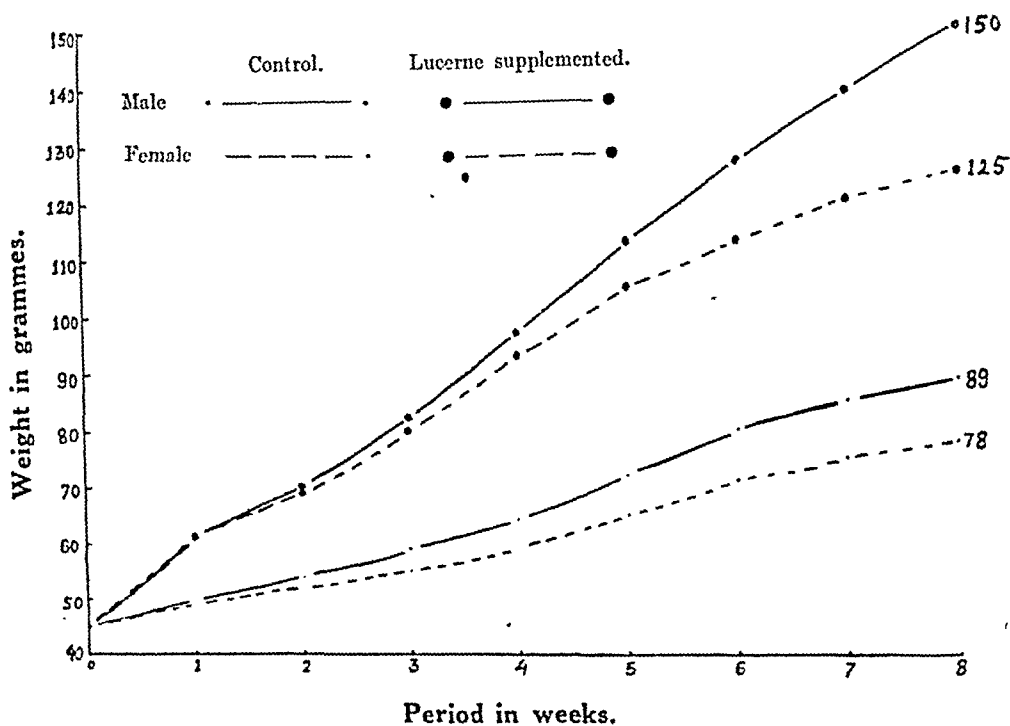
In a preliminary series rats were divided into two groups: (i) receiving the above diet alone (as control) and (ii) receiving a similar diet with the difference that 10 parts of rice were replaced by an equal weight of lucerne powder. The observations spread over a period of 8 weeks showed that the rats receiving the supplement of lucerne grew at more than double the rate of growth of the rats on the control diet.

As the above results were striking, a second series was started incorporating tamarind and chilli as part of the basal diet. This addition was made as independent experimental evidence had shown that tamarind and chilli, which normally form a part of the South Indian diet, help in increasing the rate of growth by 25 to 30 per cent over the controls when added to the original experimental diet (Krishna Murti, De and Subrahmanyam, 1948). The improved diet was prepared by adding to 500 g. of the diet a syrup representing the extract from 5.0 g. of tamarind pulp, 2.5 g. of chilli powder and 2.5 g. of salt, by soaking the tamarind pulp in water, squeezing out the juice and boiling it with chilli and salt.

Freshly weaned litter-mate rats were divided into two groups of six each:—

(i) Control group receiving the modified rice diet and (ii) experimental group receiving a diet similar to (i) with the difference that 10 per cent of the rice was

replaced by an equivalent weight of dry lucerne. The latter was cooked separately and mixed intimately with the diet. The Graph shows that for a period of 8 weeks the average weekly increase in weight for the experimental animals is about $2\frac{1}{2}$ times the growth of the control ones though the extra food intake was only 44 per cent more. The growth rate of the experimental animals would almost correspond to that of animals receiving adequate supplements of proteins, minerals and vitamins.



GRAPH.—Showing average weekly increase in weight of animals kept on lucerne diet.

With a view to evaluating the extent of benefit conferred by (i) fats and fat-soluble vitamins and (ii) vitamins of the B group and certain related substances, the following trials were made:—

Litter-mate rats were divided into 3 groups of 6 each: (a) (control) modified rice diet with lucerne as in (ii) of the previous series, (b) same rice diet *plus* the equivalent of lucerne as in (a) after four successive extractions with petroleum ether and (c) same rice diet *plus* the equivalent of lucerne after repeated extractions with 60 per cent alcohol. When extracted with petroleum ether, there was only a small loss of weight, whereas alcohol extraction resulted in about

30 per cent loss. In spite of this, the average weekly increase in weight for 8 weeks is practically the same in all the cases.

TABLE I.

Group.	WEEKLY GROWTH.		Average daily diet intake (for both the sexes).
	Male.	Female.	
(a) Control, i.e. rice diet + lucerne 10 per cent ...	13	10.3	10.1
(b) Rice diet + lucerne as in (a) after extraction with petroleum ether.	13.3	9.1	10.2
(c) Rice diet + lucerne as in (a) after 60 per cent alcohol extraction.	14.2	10.4	10.1

The animals receiving lucerne (whole as well as extracted) were under continuous health observation over a period of 4 months. It was found that liver, kidney, spleen and lungs were quite normal. There was no hæmorrhage either in the stomach or in the intestines as often observed in the case of rats receiving the poor rice diet (Krishna Murti and Subrahmanyam, 1949).

The animals from the different groups were mated and the capacity for reproduction as also the effect on the next generation were followed. The mothers were given 5 c.c. of Klim (10 per cent) daily during the lactation period only.

While all the rats on the poor South Indian diet failed to conceive even after mating for a period of 60 days, in the case of rats receiving lucerne extracted or unextracted, mating was successful for every rat. With rats on poor rice diet, resorption and death of mother during delivery have been observed by Krishna Murti and Subrahmanyam (*loc. cit.*). No such case occurred with rats receiving lucerne supplements. On an average, 7 young ones were born to each mother after about 25 days from the first day of mating.

In the group receiving supplements of whole lucerne, 40 per cent of the young ones survived; 4 per cent died young and the rest were eaten by the mothers. In the group receiving alcohol-extracted lucerne as the supplement, only 8 per cent of the young ones survived.

The weight of the males steadily rose to 213 ± 5.5 g. when 150 days old. The weight of the mothers fell by about 16 g. during the fourth week of lactation period. The weight of the young ones were 21 ± 1 g. on 21st day and 29.5 ± 1.6 g. on 28th day. Six males and 6 females about 40 g. by weight were grown for a period of 8 weeks. The average weekly increase for males was 10.6 ± 0.3 g. and 8.9 ± 0.3 g. for females. The average daily diet intake was 9.1 g.

The rats of the first generation, when mated as in the previous case, delivered about 6 young ones on an average after a period of about 27 days. Forty per cent of the young ones survived. Their growth observed for a period of 8 weeks as in the previous case was 11.6 g. for males and 9.8 g. for females.

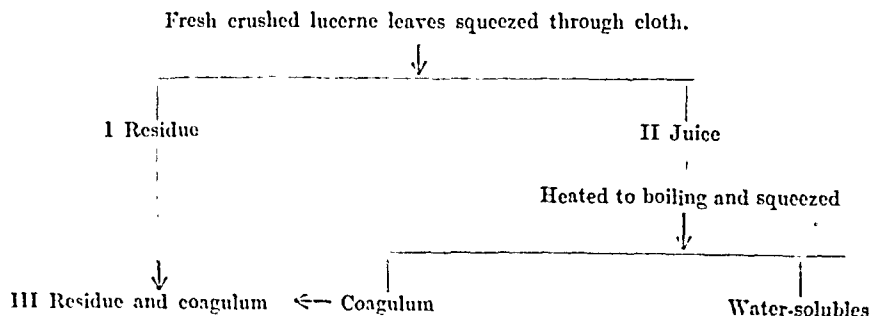
Adult rats of the first and second generation were dissected. Their lungs, liver, kidney and spleen were quite normal. There was no hæmorrhage either in the stomach or in the intestines.

The excellent growth response of the experimental animals shows that at 10 per cent level lucerne powder is an excellent supplement to poor South Indian diet for purposes of growth. It makes up for most of the deficiencies in the poor rice diet.

The rather high percentage of mortality among young ones and the cannibalism may be due to either the inability to rear the young ones which were on an average seven to each mother or some defect in the composition of the diet. A similar observation has also been made by other workers with synthetic diets which were adequate in regard to proteins, minerals and vitamins.

Though the rats of the first generation grew at a slightly lower rate than their parents, their capacity for reproduction and percentage survival of young ones was the same. The rate of growth of the rats of the second generation was slightly superior to that of their parents.

In order to evaluate the supplementary values of different fractions of lucerne, fresh tender leaves were thoroughly crushed and subjected to the following treatment :—



Fraction III was formed by combining equal parts of I and II. All the fractions were dried and powdered. For every 100 g. of whole dry leaf, 45 g. of I, 55 g. of II and 71 g. of III were obtained. As fraction II was hygroscopic, rice powder was added to make up each fraction to 100 g.

Freshly weaned litter-mate rats were divided into 4 groups of 6 each. The control group was given the previous modified diet with the further alteration that the amount of tamarind and chilli added was doubled. For the other three groups, 10 per cent of the rice of this diet was replaced by equivalent quantities of

the three powders separately. The powders were added after cooking in water-bath. Table II shows the growth response over a period of 6 weeks:—

TABLE II.

Group.	Weekly growth in g.	Daily diet in- take in g.
Control, i.e. rice diet ...	5	7.1
Rice diet + I ...	9	9.1
Rice diet + II ...	9.9	8.7
Rice diet + III ...	9.3	8.8

While each fraction shows good supplementary value, none approaches that of whole lucerne. This would show that the growth-promoting supplements are nearly equally distributed between the residue and the juice. The heat coagulum of the juice does not add to the value of the rice diet already supplemented with the residue.

For the maintenance of stock rats, a large amount of casein which is quite costly is consumed. In order to find out whether desiccated lucerne can replace casein wholly or partly, 54 male and 81 female stock rats were divided at random into 3 groups and mated, 2 males and 3 females in each cage. The stock diet of the following composition was used:—

Wheat flour 39 per cent; bajra or jowar 39 per cent; casein 7 per cent;
whole-milk powder 10 per cent; yeast 3.9 per cent; NaCl 0.5 per cent;
CaCO₃ 0.5 per cent; iron citrate 0.1 per cent.

In the first group, the rats received the stock diet which contained the entire quantity of casein. In the second group, half the casein of the stock diet was replaced by an equal weight of lucerne. In the third group, the casein was completely replaced by the same weight of lucerne. The results are shown in Table III:—

TABLE III.

Group.	MALES.		FEMALES.		Birth weight of young ones in g.	Weight of young ones on 28th day in g.
	Initial weight in g.	Increase in weight after a month in g.	Weight after delivery in g.	Decrease in weight after 28 days of lactation in g.		
Casein only ...	220	12	168	3±1	5.6	41 ±0.23
Casein half + lucerne half.	218	12	170	4±1	5.6	40.2±0.21
Lucerne only ...	221	11	157	5	5.6	38.8±0.30

There was no significant difference between the corresponding weights of the males, females and young ones in the three groups. In regard to hair and skin, the lucerne group showed slightly more roughness than that of the other two. The group receiving 50 per cent each of casein and lucerne was almost identical with that of the casein group. These observations would suggest that at least half the casein now used in stock diets can be replaced by lucerne.

SUMMARY.

1. Lucerne powder fed at 10 per cent level makes an excellent supplement to the poor South Indian diet when fed to experimental animals. The extent of supplementation would nearly correspond to that obtained by providing a complement of yeast, casein, minerals, vitamins and milk. The animals on the supplemented diet reproduce normally and rear about 40 per cent of the young ones.
2. The beneficial effect of lucerne is not exclusively due to the fats, fat-soluble vitamins, vitamins of the B group or other alcohol-soluble matter present in the lucerne.
3. The growth-promoting principles are equally distributed between the pressed juice from lucerne and the residue.
4. The heat coagulum of the juice does not effectively add to the value of the rice diet already supplemented with the residue obtained after pressing the juice.
5. In the usual stock diet as fed to rats, half the casein can be replaced by lucerne with equally good results.

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STUDIES IN PROTEIN METABOLISM.

THE INFLUENCE OF DIETARY PROTEIN ON THE URINARY NITROGEN EXCRETION.

BY

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INTRODUCTION.

THE low level of nitrogen excretion in urine by Indian adults has been reported by several workers (Wilson and Mukherjee, 1935; Ray and Ganguley, 1938; Basu and Basak, 1939; Sokhey and Malandkár, 1939; Gokhale, 1941; Niyogi, Patwardhan and Sirsat, 1941) but has been commented upon only by a few. The reported figures vary from less than 4 g. to 9.84 g. of N per 24 hours. Two suggestions have been put forward by some of the above authors to explain these findings, one is that the Indians live habitually on low protein intake and the second that since much of the protein in the Indian dietaries is derived from vegetable sources where it is enclosed in the vegetable cell wall, it is not efficiently absorbed from the intestine, thus leaving the body a smaller amount to metabolize. Very little attempt has, however, been made to provide sufficient proofs in support of these explanations. It will, therefore, be worth while to consider both points at some length.

Level of protein intake.—Sokhey and Malandkar (*loc. cit.*), Ray and Ganguley (*loc. cit.*) and Gokhale (*loc. cit.*) were of the opinion that the low urinary N in Indian subjects was due to the low level of protein intake. The available evidence obtained from family diet surveys shows, however, that the total protein intake

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per man value is not low (Wilson and Mitra, 1938; Ahmad and Gore, 1938; Shourie, 1939; Singh, 1939; Niyogi and Sukhatankar, 1939). Further, it is to be noted that Wilson and Mukherjee (*loc. cit.*) found urinary nitrogen values of 5.70 g., 6.19 g. and 8.84 g. per 24 hours on a N intake of 8.44 g., 10.77 g. and 13.25 g. respectively. Basu and Basak (*loc. cit.*) also have found 3.435 g. and 6.618 g. of urine N on intakes of 8.785 g. and 10.876 g. respectively. It does not seem correct, therefore, to assume that the low urinary nitrogen in Indians would be due to low protein intake. It is also worth while pointing out at this stage that the observations referred to in the first paragraph have been mostly made on individuals belonging to the middle classes and it is in these classes that deficiency of protein in diet hardly exists and, therefore, in considering the causes of low urinary nitrogen the question of deficiency of protein should not arise. It is admitted, however, that in the dietaries of labourers or other individuals belonging to the lowest income groups, a deficiency of protein might occur (Aykroyd and Krishnan, 1937; Krishnan, 1939; Ramalingaswami and Patwardhan, 1949).

Availability of vegetable protein.—It has already been said that a major portion of protein in typical Indian dietaries is derived from vegetable sources, chiefly from cereals and pulses. This statement can be substantiated by reference to papers on diet surveys mentioned above. To help the discussion which follows, the biological value of some of these proteins and their digestibility coefficients in rats as reported by different workers are given in Table I together with the comparative figures for some animal proteins:—

TABLE I.

Biological value and digestibility of proteins.

Source.	Biological value.*	Digestibility coefficient.	References.
	(Per cent).		
Rice	80.3 (5)	93.6	Acharya, Niyogi and Patwardhan (1942).
Wheat	67 (8 to 10)	91	Mitchell and Hamilton (1929).
Jowar (<i>Sorghum vulgare</i>) ...	83 (5)	91	Swaminathan (1937).
Bajra (<i>Pennisetum typhoideum</i>) ...	83 (5)	89	Swaminathan (1937).
Bengal gram (<i>Cicer arietinum</i>) ...	76.3 (5)	88.7	Acharya, Niyogi and Patwardhan (1942).
Red gram (<i>Cajanas indicus</i>) ...	72 (10)	75	Swaminathan (1937).
Ground-nut (<i>Arachis hypogea</i>) ...	57 (10)	90	Swaminathan (1937).
Milk (cow)	76.5 (10)	88	Mitra and Mittra (1945).
Eggs	94 (8 to 10)	100	Mitchell and Hamilton (1929).
Meat (goat)	60.4 (10)	95.2	Mitra and Mittra (1945).

* Figures in brackets refer to the level of protein fed.

It will be clear from Table I that with the exception of red gram, the digestibility of proteins from the above cereals and pulses does not compare unfavourably with that of milk and meat proteins. Although it is admitted that the above results have been obtained on rats, its applicability to human beings

will not be seriously questioned. It becomes difficult to understand, therefore, how defective digestion and absorption from the intestine, as suggested by some workers, can account for the low urinary N observed in Indian adults. Further, it must be mentioned that the proponents of the above view did not determine the faecal excretion of nitrogen; while some others who did, did not realize the significance of these determinations.

Thus, the low urinary N in Indian adults still remained unexplained. It was, therefore, considered necessary to examine the problem in greater detail. Patwardhan, Sreenivasan and Karambelkar (1948) observed in three normal adult subjects that at a constant level of protein intake, the type of protein in the diet influenced the level of urinary excretion of nitrogen. On diets based mainly on cereals with some pulse included, there was a constant retention of 2.13 g. nitrogen per day. When the protein in such diets was replaced by animal protein (from meat, milk or eggs) to the extent of 50 per cent, the urinary nitrogen increased, whereas the faecal nitrogen remained practically unchanged with the result that the daily retention of nitrogen decreased to 0.73 g.

The experiments reported in the present communication were undertaken to confirm these findings by further observations on a fresh batch of subjects under different environmental conditions and to examine some of the factors involved in this peculiar phenomenon.

EXPERIMENTAL.

Four adult subjects were selected for these experiments. They were first clinically examined and found free from any disease. In the recent past also they had not suffered from any debilitating disease. The subjects had been maintaining a constant weight over a few months before they came under observation. The daily diet of each one of them was assessed by a survey lasting over a week and their urinary nitrogen during this period determined. They were then put on diets in essence similar to their daily diets. The quantities of raw food issued were, however, weighed before cooking which in the case of subjects K. M. and S. was done in the laboratory kitchen. All the food cooked during the day was consumed under supervision. The remaining two subjects were from among the authors of this paper, and as they realized the implications of the experiment to the full, they took great care in the issue of rations, cooking and consumption of the food. A preliminary period of 7 to 10 days was allowed during which the subject adjusted himself to the new conditions. The collection of urine and faeces was made after the preliminary period. A check on the creatinine content of urine was kept throughout to ensure the proper collection of urine samples. The procedure was as follows:—

Urine and faeces were collected on two successive days, a gap of one day was then allowed followed by a second collection for another two days. If the urine nitrogen showed close approximation over these two periods, the subject was taken to have attained the stable state of excretion on the respective diet. Otherwise, collections were repeated till this criterion was satisfied. When this was achieved, protein replacement was begun. In two subjects this was done in two stages, in others in a single step, so that fifty per cent or more of the dietary protein

of vegetable origin was replaced by substitution with meat, milk or eggs either singly or in mixture. After another stabilization period, urine and faeces collection was begun till steady state was reached. At the end of the animal-protein period, the subjects were put back on their predominantly vegetarian (basal) diet and the observations repeated.

All non-perishable articles were analysed and issues were made from the same stock. Milk was re-constituted from a stock source of whole-milk powder (for subjects K. M. and S. only) which had been previously analysed. The analyses of vegetables, eggs and meat and fresh whole milk (used for subjects B. V. R. S. and P. G. T.) were carried out frequently and the averages used for calculation of N intake. The protein values for meat varied depending upon the amount of fat accompanying it. Determinations made during the week were, therefore, used for estimating the correct intake during the corresponding period.

The composition of the diets consumed by the subjects is given in Tables II to V and the data on the intake and excretion of nitrogen are illustrated in Graphs 1 to 4.

TABLE II.

Subject K. M. : Composition of the diet.

Article.	Diet I : basal, g.	Diet II : 25 per cent replacement with animal protein, g.	Diet III : 50 per cent replacement with animal protein, g.
Rice	625	582	302
Red gram	85
Cabbage	113	113	113
Potato	113	113	113
Whole-milk powder	14	14	14
Curry powder	28	28	28
Mustard	10	10	10
Jeera (cumin)	10	10	10
Onion	40	40	40
Tea	20	20	20
Meat	91	182
Sugar	56	56	142
Gingelly oil	56	56	56
Sago	200
<i>Particulars :—</i>			
Calories	3,526	3,271	3,515
Carbohydrates, g.	650	567	605
Fats, g.	69.6	80	91.4
Protein, total g.	79.2	76.4	76.7
Protein, total, as nitrogen, g. ...	12.67	12.23	12.27
Protein, animal, as nitrogen, g. ...	0.598	3.67	6.74

TABLE III.

Subject S. : Composition of diet.

Article.	Diet I : basal, g.	Diet II : 25 per cent replacement with animal protein, g.	Diet III : 50 per cent replacement with animal protein, g.
Rice	625	625	340
Red gram	85
Potato	113	113	113
Cabbage	113	113	113
Onion	40	40	40
Whole-milk powder	14	18	18
Jeera (eumin)	2.5	2.5	2.5
Mustard	2.5	2.5	2.5
Tea	20	20	20
Curry powder	28	28	28
Meat	105	200
Sugar	56	80	100
Gingelly oil	56	56	56
Sago	200
<i>Particulars :—</i>			
Calories	3,450	3,493	3,464
Carbohydrates, g.	644	621	590
Fats, g.	65.5	79.1	91.0
Protein, total g.	76.3	78.4	81.8
Protein, total, as nitrogen, g.	12.20	12.54	13.08
Protein, animal, as nitrogen, g.	0.598	3.98	7.61

TABLE IV.

Subject B. V. R. S. : Composition of diet.

Article.	Diet I : basal, g.	Diet II : 50 per cent replacement with animal protein, g.	Diet III : basal + increased calories, g.	Diet IV : diet II + increased calories, g.
Rice	405	247	405	247
Red gram	37	...	37	...
Black gram	35	5	35	5
Potato	115	60	115	60
Amaranth	85	85	85	85
Onion	65	100	65	100
Sambar powder	6	6	6	6
Chillies	5	5	5	5
Tamarind	13	13	13	13
Milk (fresh, whole)	300	690	300	690
Ghee	40	40	45	45
Sugar	15	40	80	105
Eggs	100	...	100
Sago	50	56	106
<i>Particulars :—</i>				
Calories	2,514	2,398	3,019	2,903
Carbohydrates, g.	443	358	557	472
Fats, g.	54.4	80.2	59.5	85.3
Protein, total g.	63.1	61.7	63.2	61.8
Protein, total, as nitrogen, g.	10.09	9.87	10.11	9.89
Protein, animal, as nitrogen, g.	1.58	5.61	1.58	5.61

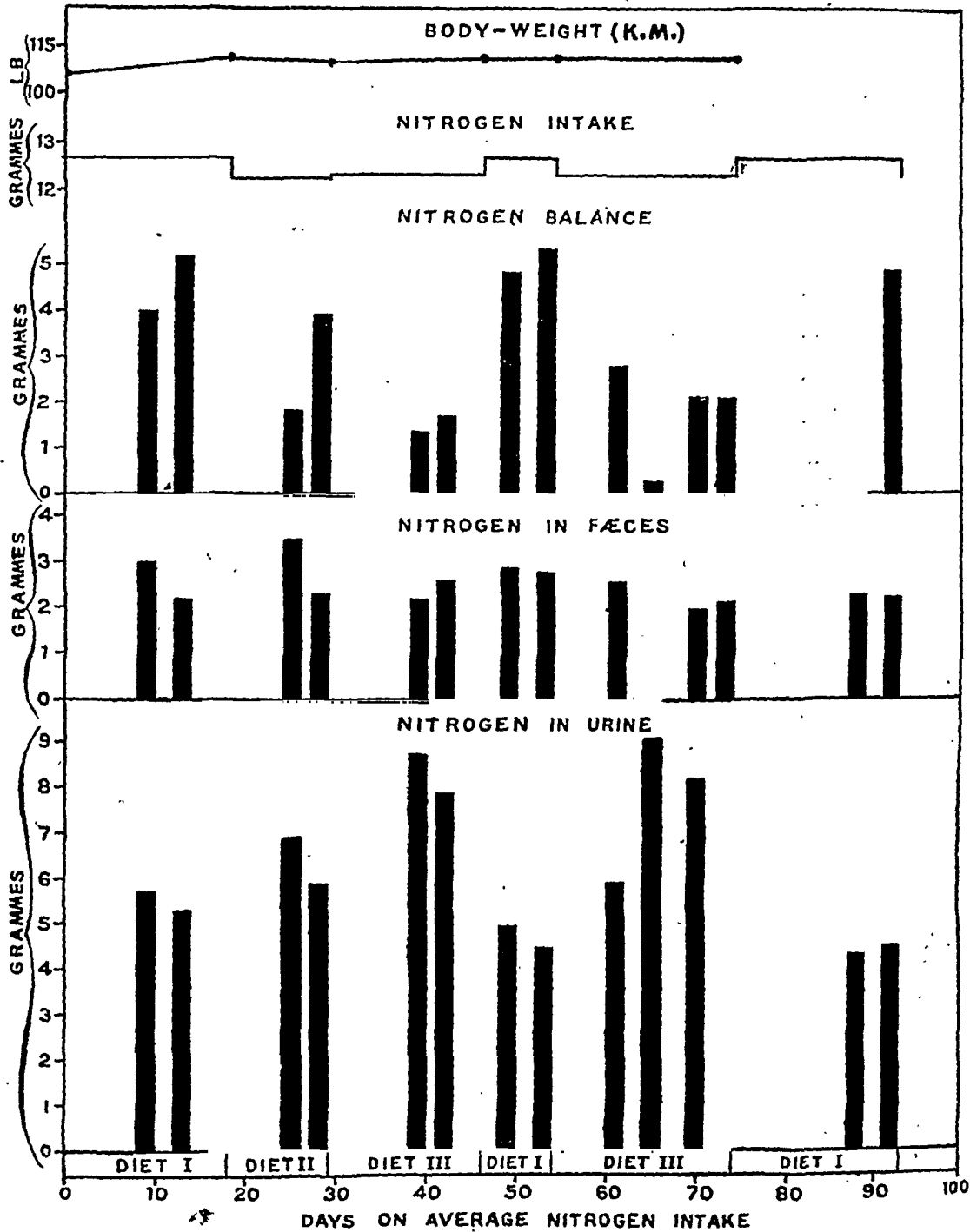
TABLE V.
Subject P. G. T.: Composition of diet.

Article.	Diet I : basal, g.	Diet II : 50 per cent replacement with animal protein, g.	Diet III : basal + increased calories, g.	Diet IV : diet II + increased calories, g.
Rice	112	112	112	112
Wheat	196	84	196	84
Red gram	70	...	70	...
Potato	112	112	112	112
Onion	28	28	28	28
Curry powder	2	2	2	2
Banana	60	60	60	60
Milk (fresh, whole)	300	300	300	300
Tea	12	12	12	12
Sugar	40	62	96	118
Ghee	28	28	33	33
Meat	130	...	130
Sago	56	56	112
<i>Particulars :—</i>				
Calories	2,142	2,064	2,611	2,533
Carbohydrates, g.	375	325	480	430
Fats, g.	40.0	58.3	49.1	63.4
Protein, total g.	61.4	60.1	61.5	60.2
Protein, total, as nitrogen, g.	9.83	9.62	9.84	9.64
Protein, animal, as nitrogen, g.	1.58	6.03	1.58	6.03

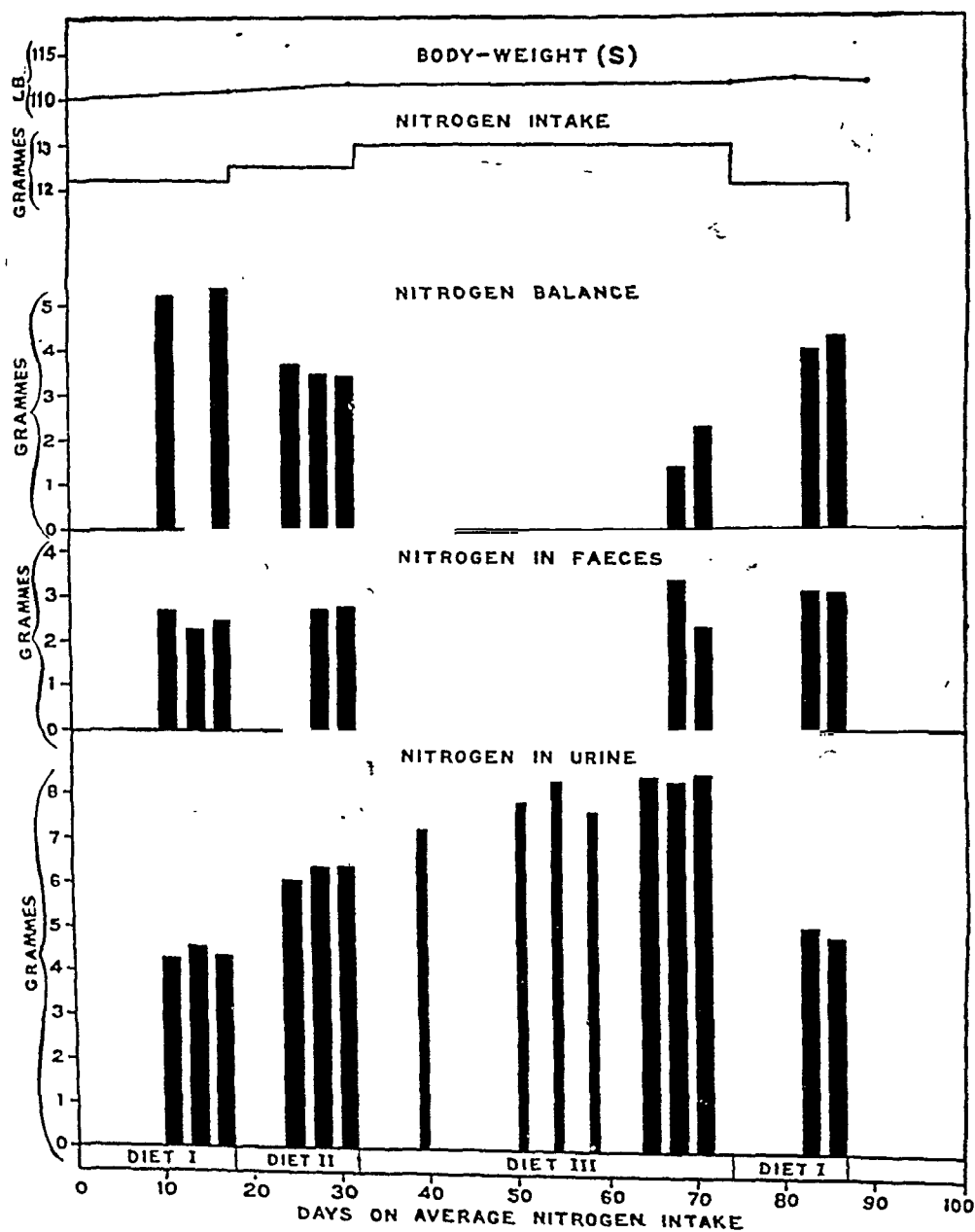
DISCUSSION.

Graphs 1 to 4 show the trends of urinary and faecal excretion of N and the balances for respective periods. The following characteristics common to all the four subjects under observation are worth noting :—

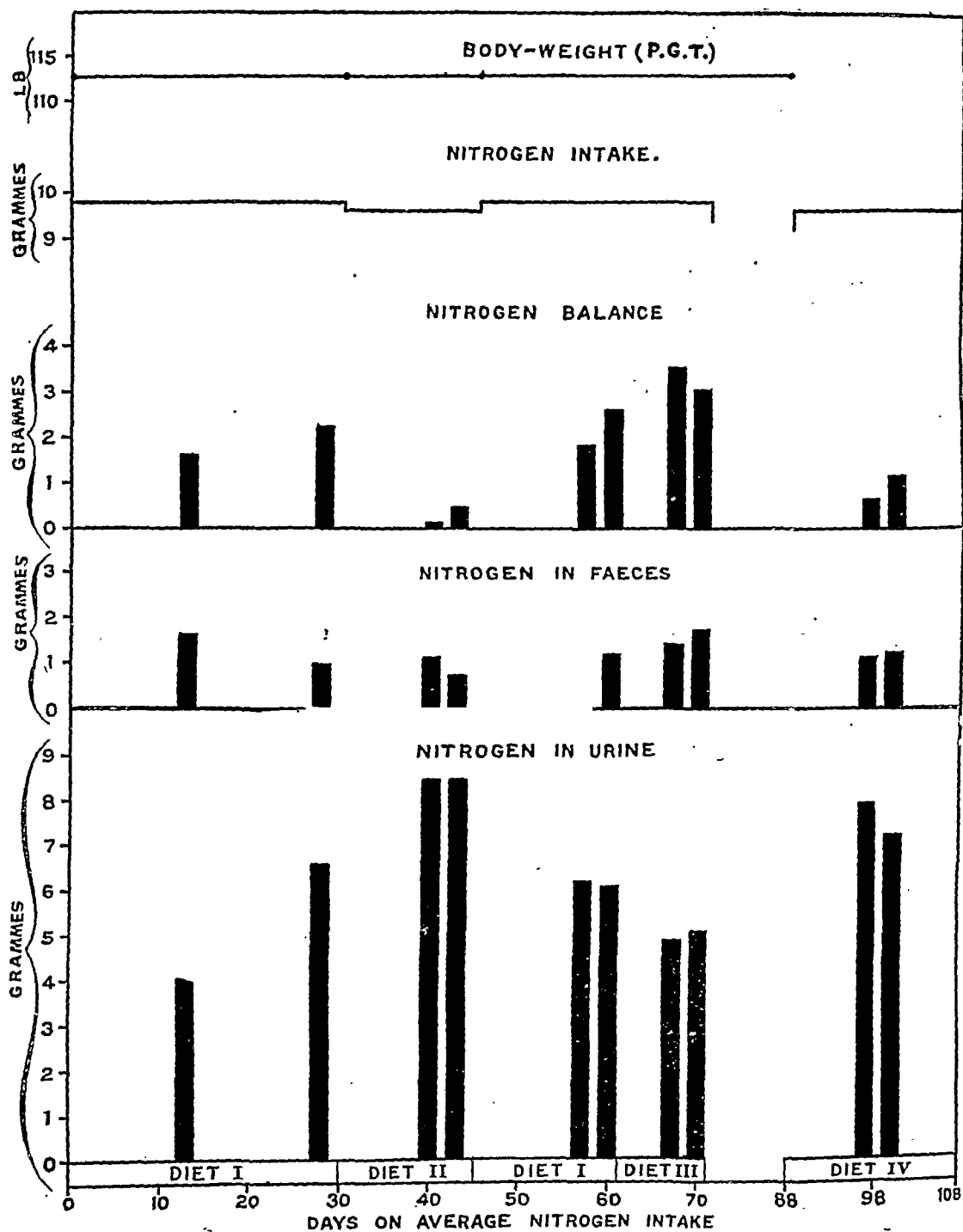
1. The urinary N was low on diets in which most of the protein was derived from vegetable sources. However, when this was partially substituted by protein



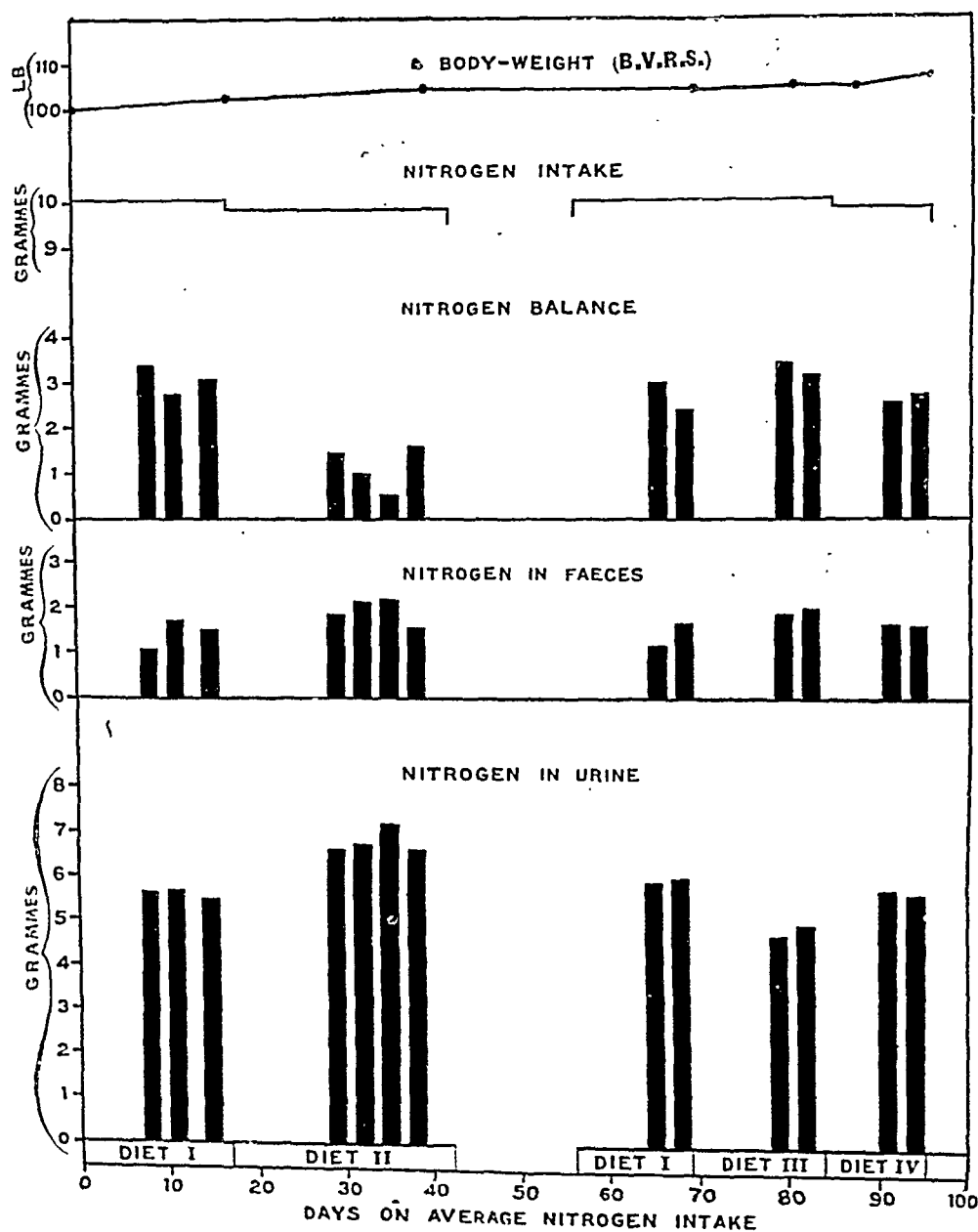
GRAPH 1.—Nitrogen balances in subject K. M.



GRAPH 2.—Nitrogen balances in subject S.



GRAPH 3.—Nitrogen balances in subject P. G. T.



GRAPH 4—Nitrogen balances in subject B. V. R. S.

from meat, milk or eggs without changing the level of intake, the urine N increased appreciably.

2. Under these conditions the faecal N output varied only within very narrow limits. The variations observed were far less than those necessary to account for the extra N in urine excreted during the animal-protein periods.

3. There was appreciable N retention in all the four subjects when they were on predominantly vegetarian diets. On partial replacement with animal protein, N retention showed a decrease, only to return to its original level after the subjects were put back on the basal diets which resembled in all cases their normal diet. These effects were the result of changes mainly in the urinary N excretion.

Nitrogen balances in individual subjects.

Subject K. M.—The calorie and total N intakes on the basal diet were 3,526 and 12.67 g. respectively. When 25 per cent replacement by animal protein was made, there was a fall in calorie intake of 255 calories and an increase of 10.4 g. in fat. During the next higher animal-protein intake (50 per cent) period, the calories were made up to the original level by the inclusion of extra sugar and sago in the diet but the fat intake showed a still further increase to 91.4 g. The question would arise whether the observed changes in urine N were due to alterations in the fat and calorie intake. It must be pointed out that although there was no difference in calorie intake between the basal diet and diet III, the corresponding urine N values per 24 hours were 5.49 g. and 8.34 g. respectively.

Subject S.—The calorie intake in this subject during all the three periods was kept approximately between 3,450 and 3,500; the fat intake, however, showed the same degree of variation as in the case of K. M. An increase of nearly 4 g. in urine N per 24 hours was observed in this subject on 50 per cent meat-protein replacement, causing a reduction in N balance from 5.40 g. to 1.84 g. per day.

Subject P. G. T.—This subject differed from the first two in two important respects. His calorie intake was only 2,142 and N intake 9.83 g. per day. Further, a larger proportion of basal dietary N was derived from animal (milk) protein. Even at this low calorie and N intake, P. G. T. was retaining 1.91 g. N per day. When animal protein formed 62 per cent of the total protein during diet II period, his urine N increased by 1.85 g., the faecal N decreased only slightly and the N retention came down to 0.26 g. On return to the basal diet, the original values for urine N and N retention were observed. In this particular subject the fat intake did not increase during the changes from one diet to another to the same extent as in other subjects for care was taken to free the meat of fatty tissue as far as possible. The observations, therefore, would suggest that a moderate increase or decrease in fat content such as was observed with subjects K. M. and S. was not responsible for the differences in urinary N excretion observed in them. Bricker, Mitchell and Kinsman (1945) report that variation of calories derived from fat ranging between 28 and 42 per cent 'did not appear to contribute to the variability of the data secured'.

Subject B. V. R. S.—The calorie intake on basal diet was about 2,500 but the N intake was approximately the same as that of P. G. T. Since this subject had conscientious objections to eating meat, the animal-protein replacement was achieved by an increase in milk and inclusion of eggs in the diet. The variation in fat intake was, therefore, similar to that observed with subjects K. M. and S. The general pattern of changes in N excretion was, however, similar to that observed in the other three subjects.

Faecal nitrogen and digestibility of dietary protein.

It has been mentioned earlier that one of the reasons for low urine N excretion in Indians which has been advanced by some observers was that the proteins from the predominantly vegetarian diet were not properly digested and absorbed, and hence an appreciable proportion of ingested protein was lost to the body. This contention is hardly borne out by facts. In the present series, the

daily faecal N observed when the steady state was reached varied in the four subjects as given in Table VI:—

TABLE VI.

Intake and excretion of nitrogen.

Subject.	MAXIMUM AND MINIMUM VALUES ON ALL DIETS.								
	INTAKE N, g.			FÆCAL N, g.			URINE N, g.		
	Maximum.	Minimum.	Difference.	Maximum.	Minimum.	Difference.	Maximum.	Minimum.	Difference.
K. M. ...	12.67	12.23	0.44	2.92	2.12	0.80	8.34	4.40	3.94
S. ...	13.08	12.20	0.88	3.09	2.35	0.74	8.43	4.45	3.98
P. G. T. ...	9.83	9.62	0.21	1.56	0.90	0.60	8.46	4.97	3.49
B. V. R. S. ...	10.09	9.87	0.22	1.96	1.40	0.56	6.87	4.86	2.01

N.B.—The maximum and minimum values quoted in Table VI were those observed when 'steady state' was reached on each diet. Earlier fluctuations have not been taken into account.

The variation of faecal N within the range given in Table VI was observed in all the four subjects without any relation to the type of diet, e.g., in some instances, the faecal N increased slightly after the subject was changed over from the basal diet to the animal-protein diet, whereas in others, the reverse has been the case. As Graphs 1 to 4 will show, this variation had little relation with the level of urinary N, for while the maximum difference in any two faecal N values of the same subjects (at steady state) was 0.80 g., the maximum difference in the urinary N excretion was of the order of 3.98 g.

There is no doubt that part of the faecal N must have been endogenous. It is difficult to say what proportion of it in the four subjects could be thus considered since no determination of N excreted on protein-free diets has been made in the present series. However, there are available a few observations made by other workers on Indian subjects. Patwardhan, Sreenivasan and Karambelkar (*loc. cit.*) found in three subjects an average of 0.9547 g. N per day and Basu and Basak (1939) have reported an average of 1.0395 g. N for two of their subjects. Both these values, although obtained in widely separated parts of India, show close approximation. Thus, it can reasonably be assumed that approximately 1 g. N in the faeces can be of endogenous origin. If this figure is subtracted from the values of faecal nitrogen observed in different subjects of the present series, one finds that the digestibility of protein (given in Table VII) shows very little difference between the basal diet and the animal-protein diet periods. The digestibility calculated for different subjects showed slightly

different values, but for each subject, the values were concordant. Thus, from these results it cannot be said that the digestibility of protein was significantly higher when animal protein in the diet formed to the extent of 50 per cent or more of the total proteins.

TABLE VII.
Digestibility of dietary protein: Four subjects.

Subject and diet.	Faecal N, g./24 hours.	Endogenous faecal N, g./24 hours.	N intake, g./ 24 hours.	Digestibility, per cent.
K. M.:				
1. Basal	2.57	1.00	12.67	87.6
2. 25 per cent meat protein ...	2.02	...	12.23	84.3
3. 50 per cent meat protein ...	2.39	...	12.27	88.6
4. Basal	2.88	...	12.67	85.1
5. 50 per cent meat protein ...	2.12	...	12.27	90.8
6. Basal	2.27	...	12.67	89.9
S.:				
1. Basal	2.35	1.00	12.20	88.9
2. 25 per cent meat protein ...	2.70	...	12.54	86.4
3. 50 per cent meat protein ...	2.82	...	13.08	86.0
4. Basal	3.09	...	12.20	82.8
P. G. T.:				
1. Basal	1.31	1.00	9.83	96.8
2. 50 per cent meat protein ...	0.90	...	9.62	100.0
3. Basal	1.45	...	9.83	95.4
4. Basal + 500 cal. from carbo- hydrates.	1.56	...	9.83	94.3
5. 50 per cent meat protein + 500 cal. from carbohydrates.	1.17	...	9.62	98.2
B. V. R. S.:				
1. Basal	1.59	1.00	10.09	94.1
2. 50 per cent egg and milk protein.	1.90	...	9.87	90.8
3. Basal	1.40	...	10.09	96.0
4. Basal + 500 cal. from carbo- hydrates.	1.96	...	10.09	90.4
5. 50 per cent egg and milk protein + 500 cal. from carbohydrates.	1.59	...	9.87	94.0

The above observations should, therefore, make it clear that the low urinary N in Indians is not necessarily the result of deficient digestion and absorption of protein from the gastro-intestinal tract. It must also have been clear from Graphs 1. to 4 that the low urinary N observed in these subjects has not been due to a low protein intake. The type of dietary protein mixture seems to determine the amount of nitrogen appearing in urine which in turn influences the nitrogen balances.

Murlin, Nasset and Marsh (1938) found in adult human beings that at the minimum level of intake of egg protein required to maintain the body in N balance, the substitution of egg protein by wheat protein caused greater loss of N through urine. This observation has been used by these authors to calculate the egg-replacement value of several proteins. Hegsted, Tsongas, Abbott and Stare (1946) also found that on an all-vegetable diet with an intake of 4.11 g. N per day, the urine N was 4.29 g. per day, whereas on diets in which meat supplied 33 per cent of nitrogen with an intake of 4.03 g. per day, the urine N was 3.87 g. per day. It will be seen that both the observations quoted above suggest a greater N loss on predominantly vegetarian diets, the differences in N excretion on vegetable- and animal-protein diets being in the vicinity of 1 g. N per day or less. Considering that the level of total N intake in the above experiments was such that it just sufficed to maintain the body in N balance, no greater variations can be expected. The greater magnitude of variations observed in the present investigation may have been due to a very much higher level of N intake. It does not, however, explain the contradiction between the present observations and those reported by the above two sets of authors. The latter have observed a greater loss of N through urine on wholly or predominantly vegetarian diets than when eggs or meat formed the dietary N either wholly or partially. In the present investigation, greater loss of N through urine has been observed in diets in which dietary protein of vegetable origin was partially replaced by that of animal origin. Whether the level of protein intake at which the respective observations were made is responsible for this discrepancy is difficult to decide at the present moment.

In view of the above, one is led to the conclusion that on predominantly vegetarian diets at levels removed from the minimum requirements and not complicated by a low calorie intake, there is appreciable retention of nitrogen. The question then naturally arises whether this N retention is real or apparent. It may be argued that before the subjects came under observation, they must have been living on diets with a negative N balance and that when they were put on the ample experimental diets, positive N balances were to be expected. This argument does not appear to be valid. It has already been mentioned that the basal experimental diet was constructed after obtaining by a diet survey an idea of the actual diets consumed by the subjects, and that the experimental basal diet represented as far as possible the normal diet of the subjects. Therefore, it is hardly likely that the subjects were suddenly put on an adequate diet. Secondly, the shifting of the N balance from high to low and back again to high value with the alteration in the type of dietary protein is an observation which it would be difficult to explain on the above assumption. For instance, the subjects K. M. and S. who showed a retention of approximately 5 g. N per day on vegetarian diets, retained only 2 g. per day on 50 per cent meat-protein diet, but on return to the basal diet, showed an increase in their N retention approximating to the original level. This observation has been repeated twice with K. M. and once with S. The subjects P. G. T. and B. V. R. S. showed similar trends although on a smaller scale.

It is conceivable that other channels of N loss (e.g. sensible and insensible, perspiration, desquamation of cornified epithelial surfaces, sebaceous secretions,

growth of keratinous tissue, and secretions of mucous membranes not thrown in the gastro-intestinal tract, etc.) have not been taken into account but they are hardly likely to be different for different dietary protein combinations' although the possibility of its being so should not be altogether ignored. Further, the subjects were allowed to reach equilibrium with their diets before collections of urine and faeces were made, this period varying with each subject as can be seen by a reference to the figures (see Graphs 1 to 4). Thus, there appears sufficient reason to conclude that the observed retentions must be real. In the first two subjects (K. M. and S.) the high calorific value of the diet may have been partly responsible for the higher N retention observed with them than in the other two subjects. This aspect is discussed in detail further on in this paper.

The observations described above raise some very important problems. Firstly, what happens to the nitrogen that is apparently retained in the body? Secondly, how can the concept underlying the technique of biological value of protein by balance-sheet method be explained in the light of these findings? Thirdly, what are the factors that influence the variation in urine N excretion when different proteins are fed at constant level much above minimum requirements?

Retention of nitrogen.

The possibility of having overlooked other channels of nitrogen loss from the body has been mentioned earlier. In this category, the only source of appreciable loss would be perspiration which according to Bricker, Mitchell and Kinsman (*loc. cit.*) could account for approximately 4 g. N per day under excessive sweating. In the present experiment, however, this channel of loss need not be a serious consideration. Coonoor is at an altitude of 6,000 feet above sea-level; the climate is usually cool when not cold and unless one indulges (which the subjects did not during the experimental period) in really strenuous exercise, sweating does not occur. Bricker, Mitchell and Kinsman (*loc. cit.*) refer to the unpublished work of Mitchell and Hamilton in which the latter found in four adult men a N loss through skin under non-sweating conditions of the order of 0.4 g. per day. It is, therefore, clear that the amount of N lost through insensible perspiration would be a small fraction of the total N unaccounted for in subjects K. M. and S.

The subjects under investigation were normal young adults whose ages varied from 24 to 30 years and who had almost stationary weights over prolonged periods. During the three months that the subjects K. M. and S. were under observation, each of them increased in weight by 2 lb. The weight of P. G. T. remained stationary throughout, although B. V. R. S. showed an increase of 2.5 lb. An attempt is made below to show with particular reference to subject K. M. that the observed weight increase would not account for the nitrogen retained. He was retaining nitrogen at different levels throughout the experimental period. If one assumes (that assumption may not be strictly valid) that between the steady states of two consecutive periods, the retention of N was an average of the retention observed in both of them, one can arrive at an approximate figure for N retention for the whole experimental period. Using this basis for calculation approximately 281 g. of N should have been retained by K. M. during the three months that he was on the experiment. If all of this was converted into proteins

and existed as such there should be 1,754 g. additional proteins in the body. If the whole of this protein were to be incorporated as component of soft tissue with an average of 75 per cent water content for the latter, the increase in soft tissue should amount to 15.5 lb., whereas the actual increase in body-weight of the subject has been 2 lb. only. The above hypothetical calculations are given to show that the observed retention of nitrogen cannot be explained by assuming its conversion to body protein. Some of it would be incorporated in the growth of keratinous tissue but a major portion might remain in a mobile form, probably that of amino-acids. It is difficult to imagine that even in this mobile form there could be continuous retention for prolonged periods. It must be assumed, therefore, that in ordinary life diets do not follow a rigid pattern and occasionally they may be such as to result in low N balances. It has not been possible to make provision for such contingency in the experiments in the form in which they were conducted. However, it might be mentioned that even in experiments over much longer periods, continued N retention has been observed although on a lower level. Grindley (1912, quoted by Bricker *et al.*, *loc. cit.*) found that in 23 young adults observed over 220 days with an average intake of 80 g. to 85 g. protein, the daily nitrogen balance averaged at 1.38 g.

Calorie intake, type of protein and nitrogen retention.

In two subjects P. G. T. and B. V. R. S. the observations were extended to study the effect of increased calorie intake of nitrogen retentions on their basal diets and after animal protein replacements (diets III and IV in Tables IV and V). The results are illustrated in Graphs 3 and 4. Diet III in these cases was essentially diet I to which 5 g. of fat and the required quantities of sugar and sago were added to give additional 500 calories approximately. The protein level was kept the same as that obtaining in diets I and II. A comparison of the relevant columns in Graphs 3 and 4 reveals that: (a) on predominantly vegetarian diet but with increased calorie intake, the urine N showed a decrease of over 1 g. per day as compared with the diet I performance, whereas the faecal N remained constant in one subject (P. G. T.) but showed an increase of 0.56 g. in another (B. V. R. S.), and (b) when animal protein was introduced in the high-calorie diet, the urine nitrogen again increased, although the increase was less than that observed at the lower calorie level (diet II). The inference which can be derived from these observations is that at higher calorie intake, protein balances were larger than at lower levels although here again the amount of urine N excreted in both cases depended upon the type of protein fed. Thus, the higher N balances in subjects K. M. and S. may have been due probably to their higher calorie intake which was in the neighbourhood of 3,500, the major proportion of calories being derived from carbohydrate, an observation which receives support from the findings of Cuthbertson and Munro (1937).

The question of the validity of determination of biological value of protein by balance experiments in adults by balance-sheet method at high level of protein intake will be dealt with in a later communication.

Finally, the important question that remains to be answered is the mechanism underlying the difference in urinary nitrogen excretion observed on the two types

of diets. Only a conjecture can be permitted at this stage and it is that there probably exist some characteristic features in the make-up of the nitrogen complex of a foodstuff which determines the metabolic outcome of the protein contained in it, the end result of which influences the urine nitrogen. It is too early to say that this difference resides in the protein, for experiments with pure proteins have not been done. Further work on this interesting problem is in progress.

SUMMARY.

Protein-metabolism studies were carried out on four adult male volunteers. Subjects were kept first on predominantly vegetarian diets and later on diets in which animal protein (from meat, milk or eggs) supplied 50 per cent or more of the protein component. The total protein content of the diets was kept nearly constant in all the periods. When the subjects reached the steady state of urinary N excretion on each diet, the following observations were recorded :—

1. The amount of nitrogen excreted in urine per 24 hours was distinctly higher on diets containing a high proportion of animal protein than on predominantly vegetarian diets. The faecal nitrogen, on the other hand, remained unaltered or showed insignificant variation.

2. As a result of changes mentioned in 1, the nitrogen retention was higher in vegetable-protein periods than in the animal-protein periods. The changes in N retention could be repeatedly demonstrated by alternating vegetable- and animal-protein diet periods.

3. The apparent digestibility of proteins during the two periods was practically of the same order.

From the observations described in the text and other considerations discussed in detail, it is concluded that the low urine N observed in Indians cannot be ascribed to a low protein intake.

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NUTRITIONAL MEGALOBLASTIC ANÆMIA : SO-CALLED PERNICIOUS ANÆMIA OF PREGNANCY.

A PRELIMINARY REPORT.

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OWING to the very high incidence and the heavy maternal and foetal mortality associated with it, the so-called 'pernicious anæmia of pregnancy' has been a subject of intense study by many workers in this country. A critical survey of the literature, however, reveals that no fixed criteria have been employed for its diagnosis. The early workers (McSwiney, 1927; Balfour, 1927) seem to have included under that name any case of severe anæmia, provided the known common causes such as loss of blood, sepsis, hookworm infection, malaria, dysentery, could be excluded. Wills and Mehta (1930), in the first serious study of the problem, defined it as a macrocytic anæmia, distinguishable from Addisonian pernicious anæmia by the incidence in younger age groups, absence of signs of cord involvement, absence of hyperbilirubinæmia as judged by the van den Bergh reaction, and by the presence of free hydrochloric acid in the gastric juice. This contention was readily accepted, though the criteria adopted to judge the presence of macrocytosis varied in different reports: the colour index (C. I.) (Wills and Mehta, *loc. cit.*; Mitra, 1931, 1937; Mudaliar and Rao, 1932; Gupta, 1932), the mean corpuscular hemoglobin (M. C. H.) (Napier and Billimoria, 1937; Napier and Mujumdar, 1938; Chatterjee and Basu, 1939), the mean corpuscular volume (M. C. V.) (Chaudhary and Manglik, 1938; Mudaliar and Menon, 1942), and

halometry (Mitra, 1937) were the different devices employed. Not always were even these criteria faithfully followed, and a careful reading of the submitted protocols shows that cases were labelled as pernicious anæmia of pregnancy even though the C. I. was lower than unity, or the M. C. V. or the mean corpuscular diameter (M. C. D.) were within the normal range or lower. This criticism applies to cases reported from the temperate climate as well (see Callender, 1944). Chatterjee (1940) noted that though the plasma-cholesterol values were low in all anæmias, the lowest readings were recorded in pernicious anæmia of pregnancy, and this, despite the fact that pregnancy *per se* produces hypercholesterolaemia. Nayer's (1942) experience was the same but no further confirmation of this work is available, either in this country or from abroad. Elliot (1944) observed that a significantly increased corpuscular fragility was an exclusive characteristic of pernicious anæmia of pregnancy, not present in 'macrocytic anæmias associated with pregnancy'. It is necessary to point out that according to Elliot (*loc. cit.*) pernicious anæmia of pregnancy, as met with in India ('tropical macrocytic anæmia of Wills'), is an anæmia merely 'associated with pregnancy' and does not show increased corpuscular fragility. Elliot's observations have not been substantiated by other workers so far and, in one instance, actually contradicted (Callender, *loc. cit.*). The most important pronouncement in recent years regarding the pernicious anæmia of pregnancy is that by Callender. A critical review of the available literature and a detailed study of her own material convinced her that the only safe criteria for the diagnosis of this anæmia are either (i) the presence of a true megaloblast in the peripheral blood or (ii) the presence of true megaloblasts in the marrow; it is essentially a megaloblastic anæmia, no matter what the peripheral blood picture be. Accessory investigations are helpful in excluding other megaloblastic anæmias. Though these observations had been made by some workers before (Lambin and de Weerd, 1939, quoted by Jones, 1943; Segerdahl, 1941, quoted by Callender, *loc. cit.*; Davidson, Davis and Innes, 1942), to Callender must go the credit for stating the case in unequivocal terms.

It is the purpose of this communication to point out that the observations of Callender are applicable to the common type of anæmia in pregnant females seen in this country and the tropics, and parading under many names: the anæmia of pregnancy (McSwiney, *loc. cit.*; Balfour, *loc. cit.*), pernicious anæmia of pregnancy (Wills and Mehta, *loc. cit.*; Mudaliar and Rao, *loc. cit.*; Chatterjee and Basu, *loc. cit.*), tropical macrocytic anæmia (Wills, 1948; Napier, 1936), anæmia of pregnancy (Gupta, *loc. cit.*), tropical anæmia of pregnancy (Editorial, *Indian Medical Gazette*, 1932), macrocytic anæmia of pregnancy (Mudaliar and Menon, *loc. cit.*), and nutritional macrocytic anæmia (Fairley *et al.*, 1938). Two points must be cleared up at the outset. Is the so-called pernicious anæmia of pregnancy, as seen in the temperate climate, identical with the similar condition encountered in this country and implied by the above names? We believe that clinically, hæmatologically and from the point of view of the natural history and the response to therapeutic agents, no distinction can be drawn between the two. Ætiologically, there is overwhelming proof that the anæmia, as met with here, is essentially nutritional (dietetic) in origin, though, at times, certain other factors (in addition to pregnancy and lactation) may precipitate its onset or even condition the deficiency responsible for it. This is true for the majority of the cases studied

in the temperate climate; the patients have been poor and subsisting on a deficient diet (Callender, *loc. cit.*). Moreover, the incidence of pernicious anaemia of pregnancy is particularly high in those regions in the West where poverty is rampant and where the general population lives on a poor diet (Callender, *loc. cit.*). The single point of difference made out is that in pernicious anaemia of pregnancy, as seen in the temperate climate, a few of the patients even though consuming a good diet yet go into this peculiar anaemic state (Stevenson, 1938, quoted by Callender, *loc. cit.*; Segerdahl, *loc. cit.*; Miller and Studdert, 1942; Callender, *loc. cit.*; Wintrobe, 1946). The nature of the extrinsic factor, or the independent haemopoietic factor supposed to be lacking in the diets of patients in our country (and the tropics) is not known and, therefore, it would be difficult to say precisely which of the diets contain it in sufficient quantity or in such a form that it is easily broken up from its combination with other substances, easily absorbed and readily assimilated. Its absence in some diets and its presence in other diets is presumed, at the moment, on mere circumstantial evidence. The distinction between the so-called pernicious anaemia of pregnancy as met with in the temperate climates and as met with in India and the tropics does not, therefore, rest on any concrete scientific proof. This statement is made with full cognizance of the fact that the deficiency responsible for the anaemia may really consist of multiple factors (Watson and Castle, 1946). The second point may be stated thus: Is there an anaemic condition peculiar to pregnancy with characteristics of its own to justify a separate existence? In our opinion, there is again no proof for this supposition. We believe that the so-called pernicious anaemia of pregnancy is identical with the so-called tropical or nutritional macrocytic anaemia in non-pregnant females and males, and, further, that this anaemia may be encountered at any age. We may add that, nevertheless, the factor of pregnancy is of importance from the point of view of the natural history (spontaneous remissions) and response to therapy (refractoriness to specific therapy seen in some patients). Taking into consideration its nutritional origin and bearing in mind that the only haematological characteristics are either the presence of a true megaloblast in the peripheral blood or a megaloblastic marrow, one of us (Bhende, 1943) has suggested that a suitable name for this anaemia is *nutritional megaloblastic anaemia*. Obviously, Addisonian pernicious anaemia would be excluded from this group. Further, the term should be restricted to the uncomplicated megaloblastic anaemia of nutritional (dietary) origin; megaloblastic anaemia of coeliac disease, tropical and non-tropical sprue, pellagra, gastro-intestinal disorders of various kinds, and fish tapeworm infection should not be included in this group.

MATERIAL AND METHODS.

During the period between May 1947 and February 1948, investigation was carried out for anemia on a series of 45 pregnant females admitted to the ante-natal wards of the Nowrosjee Wadia Maternity Hospital, Bombay. No selection was made, except that owing to the heavy demands for admission only those patients who were clinically thought to be suffering from fairly advanced anemia were admitted as indoor patients. Every case was thoroughly investigated. The obstetrical history and the history of the present and past illnesses were recorded in detail. The dietetic habits both

before and during the present pregnancy, the economic status of the family and the living condition at home were ascertained as far as possible by direct questioning. A full physical examination was made in every case. The hæmatologic investigations consisted of a detailed cytological study of the peripheral blood (including the platelet and the reticulocyte counts), the hæmatocrit determinations (Wintrobe, 1933), calculation of various relative indices and absolute values (for these a C.V. 42.0 per cent was taken as 100.0 per cent and hæmoglobin 14.5 g. as 100.0 per cent), the Price-Jones curve (Price-Jones, 1933, quoted by Whitby and Britton, 1946), the red cell fragility test (Creed, 1938; Dacie and Vaughan, 1938), the erythrocyte sedimentation rate (Wintrobe, 1933; Wintrobe and Landsburg, 1935), and examination of the sternal marrow (Bhende, 1947). In the last, the marrow-smear, at least 500 nucleated cells from 5 different fields (4 at the corners and one in the centre of the smear) were differentiated. The nomenclature followed was that of Israels (1939). Biochemical investigations included the van den Bergh reaction on the plasma, the icteric index determinations, estimation of serum proteins (Folin and Dennis, 1912; Koch and McMeekin, 1924, both quoted by Peters and van Slyke, 1932), the blood cholesterol (Leiboff, 1924, also quoted by Peters and van Slyke, *loc. cit.*), and the blood non-protein nitrogen (Folin and Dennis, *loc. cit.*; Koch and McMeekin, *loc. cit.*). The Kahn test or the Wassermann reaction was done in each case. The fractional gastric analysis (Ehrmann test-meal) with histamine administration, wherever necessary, was undertaken in all cases. The urine was examined as a routine and the urobilinogen content estimated quantitatively (Sparkmann, 1939). The fæces were examined as a routine and a special search was made for evidence of parasitic infection (Faust, 1939).

RESULTS.

As already stated, an attempt has been made in this paper to examine, in the light of diagnostic criteria laid down by Callender (*loc. cit.*), the problem of the common type of anaemia as met with in pregnant females in this country. Out of the 45 cases investigated 40 could be diagnosed as nutritional megaloblastic anaemia from the presence of a true megaloblast either in the peripheral blood or in the marrow. The findings in these 40 cases are analysed below:—

General features.—The age incidence, the relation to parity and the duration of pregnancy are shown in Table I. The living condition and the economic status may be judged from the facts that all these patients belonged to the working class, with an average income of Rs. 80 per month, and that they lived as an average family of 4 to 5 individuals (including children) in a room about 12' × 12' in the working class *chawls* of Bombay, notorious for their bad hygienic conditions. Their diet, as far as could be judged from the history, consisted of wheat bread, rice, maize, pulses, and a helping of fresh vegetables occasionally. None of them could afford protective articles of diet such as milk, fresh green vegetables and fruits. Although 24 out of 40 were non-vegetarians, they ate fish once or twice a week and meat, on the average, once a month. The general impression gained was that the diets were deficient in total proteins and animal proteins.

TABLE I.

Age incidence, parity and duration of pregnancy.

Serial number.	Age in years.	Parity.	Duration of pregnancy in months.	Serial number.	Age in years.	Parity.	Duration of pregnancy in months.	Serial number.	Age in years.	Parity.	Duration of pregnancy in months.	Serial number.	Age in years.	Parity.	Duration of pregnancy in months.
1	28	VI	8	11	30	VII	9	21	28	V	9	31	30	V	8
2	28	IV	7	12	28	VII	9	22	16	I	7	32	19	I	9
3	25	I	7	13	25	IV	8	23	18	I	5	33	28	IV	8
4	30	III	7	14	25	II	8	24	24	II	7	34	18	I	7
5	24	II	8	15	22	I	4	25	40	VIII	8	35	24	IV	7.
6	20	I	7	16	20	I	8	26	32	I	5½	36	24	II	8½
7	27	II	9	17	35	VII	8	27	38	IX	9	37	20	II	9
8	20	II	9	18	20	I	8	28	26	III	8	38	20	II	9
9	30	VI	8	19	30	IV	7	29	30	V	8	39	20	II	8½
10	28	III	7	20	23	II	7	30	26	III	7	40	25	IV	7

Signs and symptoms.—The common symptoms complained of by these patients and their frequency are listed in Table II-a and the physical findings in Table II-b.

The hæmatological findings.—The hæmatological data are presented in tabular forms (see Tables III-a and III-b). The important observations in the sternal marrow are shown in Table IV. The additional features noted in the marrow-smears may be enumerated briefly. On the whole, the smear gave an impression that the marrow was cellular and hyperplastic. All the elements of the marrow seemed to be involved. The megaloblasts were in most cases basophilic; megaloblasts with polychromatophilic or orthochromic cytoplasm were encountered in very small numbers in 12 out of 40 cases. The megaloblasts in different cases varied in size; giant megaloblasts, multinucleated megaloblasts and megaloblasts showing abnormal mitosis were seen not uncommonly. Deformed normoblasts, described by Scott (1939) as characteristic of iron-deficiency anæmia, were seen in some cases that showed a low M. C. H. C. Among the granular series, hypersegmented neutrophils, giant promyelocytes and metamyelocytes with or without atypical eosinophilic, amphophilic and azurophilic granules were identified in every case. Vacuoles in the cell cytoplasm and nuclear monstrosities with areas of rarefaction or holes in the nuclei were a common feature in these cells. The megakaryocytes were few and appeared to be depleted; they showed little platelet formation in their cytoplasm.

TABLE II.

Frequency of symptoms and signs (40 cases).

(a)	Symptoms.	Number of cases.	(b)	Signs.	Number of cases.
Easy fatigability and weakness	...	40	Pallor of mucosa and nails	...	40
Anorexia	...	28	Œdema of lower extremities	...	35
Attacks of glossitis and/or stomatitis	...	21	Glossitis	...	10
Cough	...	22	Enlarged heart and hæmic murmur	...	9
Nervous symptoms (paræsthesia)	...	11	Sepsis (teeth, pyelitis)	...	8
Diarrhœa	...	7	Koilonychia	...	7
Fever	...	11	Enlarged spleen	...	6

TABLE III-a.

Findings in the peripheral blood.

Serial number.	Hb. in g./100 c.c.	Erythrocytes in mil., per c.mm.	Packed cell vol. in c.c./100 c.c.	Total leucocytes, per c.mm.	Peripheral blood smear—nucleated erythrocytes, per 200 leucocytes.	Reticulo-cytes, per cent.	Platelets in thousands, per c.mm.
1	5.25	1.73	19.0	4,200	1 N*	0.6	...
2	4.75	1.86	22.0	6,650	1 M†	2.0	597
3	6.0	2.29	20.0	6,375	2 N	0.9	429
4	3.0	0.96	11.0	7,350	3 N	2.3	210
5	4.75	1.37	14.0	4,825	2 N	0.6	291
6	4.25	1.8	15.0	5,600	1 N	0.7	288
7	6.0	2.64	20.5	7,200	2 N	3.8	184
8	6.0	2.17	18.5	6,100	3 N	2.37	110
9	7.5	3.15	22.8	7,700	3 N	1.58	182
10	5.5	2.1	17.0	5,150	2 N	0.3	259
11	6.3	1.86	18.5	8,100	3 N	2.2	89
12	4.0	2.03	15.0	12,800	3 M	4.5	401
13	2.75	0.76	8.2	6,950	2 N	0.7	114
14	5.8	2.68	19.5	8,350	1 M	1.2	218
15	2.75	1.02	11.2	14,450	2 N	3.8	144
16	5.0	1.58	14.5	5,200	3 N	2.3	92
17	4.7	1.65	15.8	4,100	1 M	0.3	104
18	8.48	2.68	25.5	4,400	2 N	0.3	148
19	2.5	0.73	8.0	6,800	2 N	2.5	116
20	3.36	0.98	12.0	11,650	1 M	2.5	96
					5 N		
					2 M		

*N = normoblast.

†M = megaloblast.

TABLE III-a—concl'd.

Serial number.	Hb. in g./100 c.c.	Erythrocytes in mil., per c.mm.	Packed cell vol. in c.c./100 c.c.	Total leucocytes, per c.mm.	Peripheral blood smear—nucleated erythrocytes, per 200 leucocytes.	Reticulo-cytes, per cent.	Platelets in thousands, per c.mm.
21	6.1	2.20	19.7	4,400	2 N*	0.3	183
22	8.2	2.88	27.8	7,500	1 N	0.6	195
23	6.1	2.31	22.5	6,600	1 N	8.5	175
24	5.87	1.5	19.0	6,200 {	3 N	3.2	91
25	4.2	1.2	12.2	4,400	3 M†	0.8	75
26	3.85	3.0	18.0	6,500	3 N	1.7	230
27	3.4	2.10	13.8	7,000	1 N	1.8	196
28	3.22	1.12	11.0	3,250	...	0.4	122
29	4.58	1.31	14.6	10,100	1 N	1.7	157
30	6.73	3.94	25.0	3,350 {	1 N	0.6	265
31	2.7	0.97	9.0	3,250 {	1 M	1.6	52
32	4.2	1.12	12.5	7,500	1 M	1.7	170
33	4.2	1.20	12.0	7,250	1 M	0.5	101
34	3.83	2.18	15.0	4,400 {	1 N	2.4	207
35	6.78	2.20	26.0	4,900	1 M	0.4	125
36	7.20	2.97	25.0	5,350	2 N	0.4	193
37	5.6	3.33	23.5	13,400	7 N	5.2	146
38	5.84	3.95	25.0	12,300	...	1.4	620
39	7.94	3.94	30.0	13,800	1 N	2.1	450
40	3.2	0.95	11.0	5,650 {	3 N	0.9	94
					2 M		

*N = normoblast.

†M = megaloblast.

TABLE III-b.

Relative indices and absolute values.

Serial number.	C. I.	M. C. H. $\gamma\gamma$.	M. C. V. μ^3 .	M. C. H. C., per cent.	PRICE-JONES CURVE.			M. C. A. T. μ .	M. C. D./M. C. T.	ERYTHROCYTE FRAGILITY AS PER CENT OF NaCl.			E. S. R. mm./hour.
					M. C. D. μ .	$\delta \mu$.	V, per cent.			Start (5 per cent).	End (95 per cent).	M. C. F. (50 per cent).	
1	1.04	30.3	109.8	27.6	0.48	0.40
2	0.88	25.5	118.0	21.6	0.48	0.40
3	0.9	26.2	87.3	30.0	0.44	0.34
4	1.09	31.2	114.5	27.2	0.48	0.40
5	1.19	34.6	102.1	33.9	0.44	0.36
6	0.81	23.6	83.3	28.3	7.2678	0.6846	9.419	2.007	3.62	0.36	0.32	0.355	...
7	0.76	21.9	77.6	28.3	6.888	0.5866	8.561	2.085	3.309	0.44	0.36	0.404	16
8	0.98	27.6	85.2	32.4	7.412	0.6862	9.258	1.974	3.754	0.48	0.34	0.415	31
9	0.82	23.8	72.4	32.9	6.948	0.6098	8.776	1.824	3.811	0.50	0.34	0.424	21
10	0.9	26.2	80.9	32.3	7.3512	0.6362	8.654	1.905	3.859	0.44	0.30	0.374	21
11	1.16	33.8	99.4	34.0	7.7816	0.6439	8.269	2.09	3.724	0.46	0.36	0.414	13
12	0.68	19.7	73.9	26.6	6.9348	0.6084	8.774	1.956	3.545	0.44	0.32	0.395	10
13	1.24	36.1	107.9	33.5	7.914	1.0856	13.71	2.192	3.611	0.46	0.32	0.407	30
14	0.74	21.6	72.7	29.2	7.0728	0.8532	12.34	1.85	3.822	0.46	0.32	0.394	14
15	0.93	26.9	109.8	24.5	7.6988	0.6412	8.329	2.358	3.264	0.44	0.32	0.386	21
16	1.04	31.6	91.1	34.4	7.1424	0.652	9.182	2.274	3.142	0.44	0.30	0.375	16

17	0.98	28.4	95.7	29.6	7.6217	0.7345	9.636	2.109	3.614	0.44	0.30	0.375	21
18	1.03	31.4	95.1	32.9	7.5352	0.598	7.935	2.133	3.524	0.46	0.32	0.403	26
19	1.2	34.4	109.6	31.2	8.08	1.18	14.61	2.137	3.816	0.46	0.32	0.416	24
20	1.3	34.6	122.4	28.0	8.099	1.211	14.95	2.375	3.409	0.46	0.32	0.406	11
21	0.91	26.6	83.0	30.9	7.013	0.5418	7.725	2.201	3.186	0.46	0.30	0.396	16
22	0.97	28.4	95.4	29.8	7.592	0.6443	8.48	2.107	3.602	0.46	0.30	0.413	30
23	0.91	26.4	97.4	27.1	7.947	0.6337	7.975	1.964	4.046	0.42	0.32	0.374	31
24	1.3	39.3	126.6	30.8	8.145	0.7605	9.337	2.43	3.352	0.48	0.34	0.414	26
25	1.2	35.0	101.6	34.4	7.5155	0.8046	8.046	2.675	2.808	0.44	0.32	0.376	21
26	0.14	12.8	60.0	21.4	6.815	0.8077	11.86	1.645	4.142	0.44	0.30	0.355	10
27	0.53	15.5	63.01	24.6	6.8825	0.6067	6.997	1.693	4.003	0.48	0.32	0.406	14
28	0.99	28.7	97.3	29.2	7.263	0.6707	9.315	2.335	3.108	0.44	0.40	0.415	38
29	1.2	34.9	111.4	31.3	7.4505	0.7422	9.962	2.558	2.916	0.46	0.36	0.355	8
30	0.6	17.5	65.6	26.9	6.5275	0.8612	13.26	1.972	3.329	0.42	0.28	0.398	30
31	0.96	27.8	92.7	30.0	7.028	0.646	9.199	2.378	2.957	0.40	0.28	0.376	22
32	1.20	37.5	111.6	33.6	8.189	0.8135	9.938	2.119	3.864	0.42	0.28	0.386	21
33	1.12	32.5	93.05	35.0	6.9045	0.7605	10.72	2.435	3.497	0.44	0.26	0.375	40
34	0.6	17.5	68.8	25.5	6.368	0.8425	13.22	1.907	3.341	0.44	0.30	0.393	22
35	1.02	29.6	113.5	26.07	7.5905	0.6292	8.288	2.51	3.024	0.46	0.30	0.305	21
36	0.84	24.5	84.1	29.5	7.0395	0.715	10.16	2.102	3.257	0.46	0.32	0.374	12
37	0.57	16.8	70.5	23.4	6.836	0.6155	9.149	1.921	3.559	0.48	0.28	0.413	18
38	0.51	14.7	64.4	24.3	6.422	0.5502	8.570	1.58	4.065	0.46	0.28	0.404	20
39	0.69	20.1	76.1	26.4	6.8045	0.587	2.039	3.373	0.46	0.34	0.34	0.436	18
40	1.15	33.6	115.7	29.1	7.2905	0.5992	6.984	2.771	2.63	0.46	0.34	0.436	18

TABLE IV.

The marrow findings.

Serial number.	Hæmocyto- blasts, per cent.	Proerythro- blasts, per cent.	MEGALOBLASTS, PER CENT.		NORMOBLASTS, PER CENT.		
			Hb.- ized.	Non-Hb.- ized.	I.	II.	III.
1
2	0.0	0.0	0.0	3.5	2.1	29.4	14.5
3	0.0	0.0	0.0	21.0	3.5	20.8	2.2
4	0.0	0.2	0.0	23.0	3.5	13.4	1.2
5	0.4	0.4	0.0	9.6	5.8	26.2	1.6
6	0.4	0.0	0.0	2.8	3.2	16.6	2.0
7	0.0	0.0	0.0	1.0	2.0	21.2	3.6
8	0.2	0.8	0.0	24.8	2.4	16.0	3.2
9	0.2	0.4	0.0	18.0	1.6	18.0	4.8
10	0.8	1.0	0.0	28.8	4.8	15.6	1.0
11	0.0	0.3	0.1	18.3	8.3	16.0	4.6
12	0.0	0.2	0.0	3.8	6.1	22.0	10.0
13	0.2	0.4	0.0	20.8	7.6	22.6	6.0
14	0.6	1.0	0.0	22.4	4.0	20.0	1.2
15	0.0	0.0	0.0	4.0	0.8	12.3	49.8
16	0.35	0.0	0.95	19.9	8.9	17.9	3.2
17	0.4	0.0	0.0	17.5	8.3	17.7	4.8
18	0.25	0.0	0.12	15.5	2.25	20.5	3.53
19	0.0	0.0	0.2	14.4	4.0	23.8	1.6
20	0.7	0.0	2.55	19.1	3.55	18.85	7.32
21	0.12	0.12	0.25	8.25	1.75	19.26	3.6
22	0.0	0.5	0.0	4.0	3.3	38.2	14.0
23	0.13	0.65	0.0	3.25	3.5	53.37	7.8
24	0.3	0.5	0.83	28.33	5.7	17.9	3.74

The values are expressed as per cent of total nucleated cells.

TABLE IV—concl'd.

Serial number.	Hæmocyto-blasts, per cent.	Proerythro-blasts, per cent.	MEGALOBLASTS, PER CENT.		NORMOBLASTS, PER CENT.		
			Hb.-ized.	Non-Hb.-ized.	I.	II.	III.
25	0.65	0.83	0.0	15.0	3.0	26.0	4.67
26	0.0	0.0	0.0	5.8	5.0	35.0	10.44
27	0.0	0.5	0.0	0.83	0.66	34.8	7.4
28	0.75	0.18	1.1	16.65	9.8	14.8	2.57
29	0.3	0.65	0.0	23.3	20.1	12.3	8.95
30	2.5	0.6	2.8	23.2	1.2	12.8	9.6
31	0.4	0.0	4.4	20.0	9.7	5.5	8.8
32	0.6	0.65	0.65	17.7	16.5	9.5	5.6
33	0.3	2.8	0.35	26.0	7.3	14.8	7.0
34	0.3	0.14	0.0	11.5	2.2	28.5	10.46
35	0.0	0.0	0.0	7.75	2.0	22.0	9.05
36	0.14	0.7	0.0	18.2	4.5	30.5	5.96
37	0.4	0.14	0.0	5.4	3.4	54.52	9.1
38	0.0	0.12	0.0	2.0	2.2	42.5	8.91
39	0.25	0.25	0.0	3.0	3.7	35.0	15.01
40

The values are expressed as per cent of total nucleated cells.

Biochemical findings.—The van den Bergh reaction gave an indirect positive reading in 11 cases; in all these cases the icteric index was raised. The values fluctuated between 7 and 9 units in 9 cases; in 2 cases the icteric index was 12 and 15 units respectively. In the rest, the reaction was negative and the icteric index was between 2 and 7 units. The hyperbilirubinæmia (as judged by the positive van den Bergh reaction and an elevated icteric index) was not accompanied by urobilinuria. The values for urine urobilinogen, as determined by Sparkmann's (*loc. cit.*) method, were well within the normal range in all cases. Estimation of the serum proteins revealed hypoproteinaemia in 33 cases. Blood-cholesterol level was between 100.0 mg. and 200.0 mg. per 100 c.c. in the majority of the cases; in 2 cases the reading was 228.5 mg. and 246.1 mg. respectively and in another 2 cases 80 mg. The figures for the non-protein nitrogen ranged from 20.3 mg. to 34.2 mg. per 100 c.c. Gastric analysis showed normal acidity.

in 7, hyperchlorhydria in 2, hypochlorhydria in 29, and a histamine-fast achlorhydria in 2 cases. Routine urinalysis showed albuminuria in 21 cases; in 16 it could be described as traces of albumin, in 5 as 1+.

Other findings.—The blood Kahn test or Wassermann reaction was positive in 6 cases of the series. Routine examination of faeces revealed that parasitic infection was not uncommon among our patients. Thus, ankylostome ova were present in 7 cases; ova of *A. lumbricoides* in 8; ova of *T. trichuria* in 4; cyst of *E. histolytica* in 4 and cyst of *G. lamblia* in 1.

COMMENT.

The clinical picture of nutritional megaloblastic anaemia (in pregnancy) that emerges from the analysis may be stated thus: The age incidence is earlier than in Addisonian pernicious anaemia. The anaemia is usually insidious in onset and is usually fully developed in the third trimester of pregnancy. Some cases, however, show a quick onset and a rapid evolution; precipitating causes like sepsis or persistent vomiting may be present in such cases. The vast majority of the patients are poor; low economic status, bad living conditions, and a defective diet (especially lacking in proteins) are their lot. Compared to the deteriorated state of the blood, there is a paucity of symptoms. The common symptoms include general weakness, oedema of the ankles, attacks of glossitis (indistinguishable from that of Addisonian pernicious anaemia, except that a smooth glazed tongue or atrophic glossitis does not occur), and stomatitis, gastro-intestinal disturbances (anorexia, diarrhoea, vomiting) and fever. Paræsthesias in the limbs are not uncommon. Other symptoms include those common to all severe anaemias. Among the commoner physical signs, in addition to those of any severe anaemia (like pallor, low blood pressure, and a hæmic murmur), may be mentioned oedema of the ankles and enlargement of the spleen. Sub-acute combined degeneration of the spinal cord was not encountered and is said not to occur in these cases (both tropical and those of the temperate climate). Degazon (1945), however, has recorded a case in a pregnant female with a spontaneous recovery after delivery. It will be seen that no symptom is specific and no physical sign diagnostic. In every case a full hæmatological study is, therefore, imperative.

The anaemia is fairly advanced by the time the patient seeks advice. The blood picture, in general, conforms closely to the description of dimorphic anaemia as given by Trowell (1942). In uncomplicated cases, the leucocyte count is normal or there is leucopenia. The platelets are reduced. No cases showing purpura associated with this thrombocytopenia have been encountered, but Fairley *et al.* (*loc. cit.*) have noticed this association in 25 per cent of their cases and, in some of their patients, purpura was the presenting symptom.

The reticulocyte count is usually low. In our series we have been unable to correlate the occurrence of splenomegaly, hyperbilirubinæmia and reticulocytosis (except in one case where malarial parasites could be demonstrated), but such cases have been described in this country (Napier, 1939), in Macedonia (Fairley *et al.*, *loc. cit.*) and in Africa (Trowell, 1943) under the name of nutritional macrocytic anaemia hæmolytica. Precedent chronic malaria is considered as an

etiological factor (in addition to dietetic deficiency) in such cases. The values for the C. I., M. C. H., M. C. V., M. C. H. C. and the M. C. D. are extremely variable and no reliance could be placed on them for correct diagnosis. Adopting the figures of Price-Jones *et al.* (1935) for the normal range of M. C. V. and M. C. H. C., our cases could be classified into different groups as shown in Table V, wherein the correlation of these values with the criteria adopted for the diagnosis of nutritional megaloblastic anaemia has been shown. It will be seen that if one were to rely on macrocytosis (as determined by M. C. V.) for the diagnosis of this anaemia, as is usually advocated, only 17 of the 40 cases would have been spotted out. The 15 cases showing normocytosis and 8 cases showing microcytosis would have been missed, as without a meticulous search for a megaloblast in the peripheral blood or the demonstration of megaloblast in the marrow, they would not have been diagnosed as nutritional megaloblastic anaemia. It may be emphasized that even microcytosis in the peripheral blood may not be incompatible with the diagnosis of nutritional megaloblastic anaemia. Such cases have been reported by others (Lambin and de Weerd, *loc. cit.*; Segerdahl, *loc. cit.*; Davidson *et al.*, *loc. cit.*; Foy and Kondi, 1943; Woolf and Limarzi, 1945). Similarly, the M. C. D. in nutritional megaloblastic anaemia may be slightly raised, be within the normal range, or may be even lowered. Anisocytosis and poikilocytosis are common in the blood-smear but the Price-Jones curve shows that in nutritional megaloblastic anaemia the values for standard deviation and coefficient of variation are below those given for Addisonian pernicious anaemia (*see* Table III-b).

TABLE V.

The relationship between the type of anaemia and the findings of megaloblast in the peripheral blood and the marrow.

Class.	Number of cases.	Number of cases showing megaloblast in peripheral blood-smear.	Number of cases showing megaloblastic marrow.
Macrocytic, orthochromic ...	10	10	10
.. hypochromic ...	7		6
Normocytic, orthochromic ...	13	3	13
.. hypochromic ...	2		2
Microcytic, orthochromic ...	1	2	1
.. hypochromic ...	7		7

Macrocytosis as judged by M. C. V.: normal range 75-744 c.μ to 96-096 c.μ.

Hæmoglobin concentration as judged by M. C. H. C.: normal range 28-17 per cent to 34-35 per cent.

In one case sternal puncture was refused but the diagnosis could be made by the demonstration of megaloblasts in the peripheral blood-smear.

Though the figures for the mean corpuscular average thickness (M. C. A. T.) are higher than 2.545μ , the upper limit of normal (Price-Jones *et al.*, *loc. cit.*) in 3 cases (see Table III-b), the figures for the ratio $\frac{M. C. D.}{M. C. A. T.}$ which is a better index to spherocytosis fall within the normal range in all our cases. Thus, we are unable to confirm the contention of Fairley *et al.* (*loc. cit.*) and Chatterjee (*loc. cit.*) that in this anaemia there is spherocytosis, or a disproportionate increase in the red cell thickness.

Nor did we find any significant increase in the erythrocyte fragility which Elliot (*loc. cit.*) claims to be diagnostic of pernicious anaemia of pregnancy of the temperate climate (see Table III-b). Our findings fully support the statement of Callender (*loc. cit.*) that the only diagnostic feature of the peripheral blood is the presence of a true megaloblast.

As megaloblasts in the peripheral blood, even in a severe case, may be few and are, therefore, apt to be missed unless a prolonged careful search of the smear is undertaken, in every case of anaemia, hæmatologic investigations are incomplete without the examination of a marrow-smear. In all our cases (except one, where the sternal puncture was refused) megaloblasts were unequivocally demonstrated in the marrow. Ehrlich's hæmoglobinized megaloblasts are rare or few in these cases, but the significance of this observation in differentiating this anaemia from Addisonian pernicious anaemia cannot be dogmatically stated. In most of these cases megaloblasts (basophilic) were present in plenty (see Table IV) and could easily be spotted out at a glance. In some cases, probably very early or those which may have had incomplete specific treatment, a careful and often prolonged search may have to be undertaken for their detection. It has been realized that it is not always easy to identify these cells and the difficulty correspondingly increases when they are present in very small numbers. In such circumstances the aberrations in the granular series are a good aid. Zuelzer, Newhall and Hutaff (1947), studying the marrow changes in the megaloblastic anaemia of infancy, concluded that these abnormalities of the leucocytes were the earliest changes in megaloblastic transformation of the marrow. The importance of examination of the marrow in every case cannot be over-emphasized.

Hypoproteinaemia was found in 33 cases of this series but cannot be considered diagnostic for this anaemia. Seven cases gave normal values for plasma-proteins. We cannot agree with Callender (*loc. cit.*) that a normal reading for the plasma-proteins rules out dietetic deficiency as far as this anaemia is concerned. Our results of blood-cholesterol estimation show that this constituent is depleted in these cases—considerably—as normally in pregnancy there is hypercholesterolaemia. At the same time, we could not confirm, in our series, the very low values obtained in these patients by Chatterjee (*loc. cit.*) and Nayer (*loc. cit.*). In any case we do not think that this finding is a valuable diagnostic feature or specific for this anaemia. Hyperbilirubinaemia is seen in some cases. Its existence has been confirmed by direct plasma-bilirubin estimations. It is seen both in patients from zones where malaria is endemic and in regions where it is unknown. Hyperbilirubinaemia, if accompanied by a high reticulocyte count, would be diagnostic of the hæmolytic variant of nutritional megaloblastic anaemia. By itself it should not be so considered. Similarly, the original contention of Wills and Mehta (*loc. cit.*) that hyperbilirubinaemia is a point against the diagnosis

of tropical macrocytic anæmia and in favour of a diagnosis of Addisonian pernicious anæmia is no more tenable. Wills (*loc. cit.*) has accepted this modification. Gastric analysis shows the presence of free hydrochloric acid in the majority of these cases of nutritional megaloblastic anæmia. A histamine-fast achlorhydria is found in some cases both in the pregnant females and males (Fairley *et al.*, *loc. cit.*; McRobert *et al.*, 1940; Bhende, 1942; Davidson *et al.*, *loc. cit.*). With treatment of the anæmia the acid returns in some of the cases. The presence of a histamine-fast achlorhydria should not be considered as a point negating the diagnosis of nutritional megaloblastic anæmia. Lastly, in this country, examination of the fæces for parasitic infection should never be neglected. Not a few cases are complicated by a coincident ankylostome infection; this was present in 7 of our cases and has been reported by others.

In conclusion, it may be pointed out that in the light of the re-orientation of the views regarding the diagnostic criteria for this anæmia many of the previously expressed opinions on the different aspects of this condition will have to be re-assessed.

SUMMARY.

1. A preliminary report of 40 cases of so-called pernicious anæmia of pregnancy is submitted.

2. A detailed analysis of these cases shows that no symptoms are specific and no physical signs diagnostic for this anæmia. The only reliable criteria for the diagnosis of this condition are: (i) the presence of a true megaloblast in the peripheral blood and (ii) the presence of a true megaloblast in the marrow.

3. These criteria, originally laid down by Callender for the pernicious anæmia of pregnancy as met with in the temperate climate, are applicable to the similar condition seen in this country.

4. It is argued that clinically, hæmatologically, and, ætiologically, the pernicious anæmia of pregnancy as met with in the West is identical with the similar condition met with in India and the tropics.

5. A new term *nutritional megaloblastic anæmia* is proposed for this anæmia. It is pointed out that this anæmia can also be encountered in non-pregnant females and males of any age.

6. The importance of a full hæmatological investigation in every case of anæmia is stressed.

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ON REACTIONS FOLLOWING RE-VACCINATIONS WITH
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EARLY in 1940, at the suggestion of the Ministry of Health in England, conveyed through the then Public Health Commissioner with the Government of India, it was decided to undertake a field investigation to ascertain whether in the presence of smallpox, vaccinia virus cultivated on the chorio-allantoic membrane of developing chick embryos would equal, in its protective value, the calf lymph in routine use in the Province. Twenty-six villages in the vicinity of the Institute on the main lines of communication were chosen for the study. In each village, the available child population for primary vaccination at each visit was divided into two groups: one was vaccinated with the membrane virus (hereinafter called for convenience 'membrane lymph') and the other with calf lymph. There was no bias in the selection of cases, the grouping being done on the basis of alternate cases. It was the intention to study the relative incidence of attacks and deaths in the two groups in the event of an epidemic of smallpox occurring in any of the villages.

The vaccinations were performed by the regular vaccination staff of the Public Health Department and the results obtained checked periodically by a team from the Institute, thus assuring uniformity and accuracy of data. The operation of vaccination was performed with the so-called 'rotary lancet' in common use throughout India, by superficial scarification of the skin through a dose of vaccinia

virus placed on it. One end of the lancet has a circular disc with five pointed scarifiers which on rotation make a circular cut about 6 mm. in diameter. The other end of the lancet is provided with a small scoop with a central hole in it to facilitate the removal of lymph from the lymph vial. It was the practice in primary vaccination to supply the vaccine at four separate sites, two on each arm. Uniformity of the vaccination area was thus assured in all cases by this technique.

The reactions following vaccination with membrane lymph were, as a general rule, milder than in the case of calf lymph though the evolution of the lesion was characteristic of vaccinia. The case success rates were also lower, being 89.2 per cent as compared with 99.1 per cent in calf lymph. It would, therefore, be correct to say that in a general sense the membrane lymph was less 'potent' than calf lymph. It should, however, be stated that a higher percentage of 'takes' with the membrane virus has not been recorded by other observers.

The experiment lasted for 5 years and the results of the comparative study are being reported separately. At the end of 4 years, during which period the experimental group of villages escaped any severe outbreak of smallpox, it was, however, decided to study the effect of re-vaccination in the children primarily vaccinated during the first year with the two types of lymph. In order, however, not to vitiate the set up for the original purpose of the study, namely, the comparative efficacy of the two types of vaccine in the presence of smallpox, the re-vaccinations were performed with the homologous type of vaccine. Thus, children who were primarily vaccinated with calf lymph were re-vaccinated with calf lymph and the other group with the other lymph. For purposes of comparison, however, re-vaccination with heterologous type of lymph was carried out subsequently in a further series of cases. At a later stage, the effect of re-vaccination six months after 'primary' vaccination was studied in yet another group. The present note deals with the observations made in the course of this study in re-vaccination.

The operation of re-vaccination was performed in exactly the same manner as primary vaccination except that the vaccine was inserted at only two sites, usually on the left forearm. The results of re-vaccination were observed after 24 hours, 72 hours and 120 hours, and again after 8 days. In recording the nature of the reaction following re-vaccination, the criteria mentioned by Leake (1927) were generally followed. Thus, the occurrence of a reaction which manifests itself as an area of redness at the site of vaccination, appearing and reaching its maximum within 72 hours, fading away without any subsequent change was recorded as an 'immune reaction'. This was almost always accompanied by a slight elevation of the skin over the vaccinated area. A reaction in which the lesion passes through all the stages of 'primary' vaccinia but in a shorter time, the maximum being reached and passed between the 3rd and 7th days was considered as an 'accelerated reaction' and recorded as a 'modified take'. The appearance of a vesicle was considered essential to define this reaction. Frank 'takes' characteristic of primary vaccinia were recorded as such and cases which did not show any reaction at all were regarded as 'failures'. No attempt was made to repeat the operation of vaccination in the case of 'failures', nor any controls with killed vaccine included in the series.

The results of re-vaccination of children, primarily vaccinated with success four years previously, are summarized in Table I. These include observations on (i) a group of 350 children who were primarily vaccinated with membrane lymph and re-vaccinated with membrane lymph; (ii) 373 children primarily vaccinated with calf lymph and re-vaccinated with calf lymph; (iii) 66 children in whom 'primary' vaccination was with membrane lymph and re-vaccination with calf lymph; and (iv) 65 children in whom the 'primary' vaccination was with calf lymph and re-vaccination with membrane lymph.

TABLE I.

Results of re-vaccination of children primarily vaccinated 4 years previously.

Primary vaccination :—	Membrane.		Calf.		Membrane.		Calf.	
Re-vaccination :—	Membrane.		Calf.		Calf.		Membrane.	
	Number.	Per cent.	Number.	Per cent.	Number.	Per cent.	Number.	Per cent.
'Failure' ...	113	32.3	13	3.5	1	1.5	0	0
'Immune reaction' ...	186	53.1	154	41.3	13	19.7	61	93.8
'Accelerated reaction' ...	39	11.1	128	34.3	14	21.2	3	4.6
'Takes' ...	12	3.4	78	20.9	38	57.6	1	1.67
TOTALS ...	350		373		66		65	

It will be seen that distribution of the types of response to re-vaccination is different in the different groups. The largest proportion of failures occurs in the group where both primary and re-vaccinations were done with membrane lymph, Leake (*loc. cit.*) has stated that with the use of a potent lymph for re-vaccination there should be no 'failure' if sufficient precautions are taken to ascertain the results as early as possible. As already mentioned, the first inspection of the result of re-vaccination was made as early as 24 hours after the operation. Even so, as many as 113 children out of 350 in this group, i.e. 32.3 per cent, failed to show any reaction. While it is true that these cases were not tested by a repetition of the

operation of vaccination, the marked difference from the failure rates in the other groups must be considered significant. Faults in technique were not probably responsible for the high failure rate, as vaccinations performed at the same time with calf lymph have resulted in a much smaller proportion of failures, viz., 3.5 per cent in a group of 373. The difference is even more striking when these results are compared with the results in the 3rd group of 66 children primarily vaccinated with membrane lymph and re-vaccinated with calf lymph where only one child failed to show any reaction. Also, primary vaccinations in children (not included in the series) done at the same time and with the same batches of membrane lymph have yielded the usual case and insertion success rates. It would, therefore, appear that the large percentage of 'failures' in re-vaccination with membrane lymph is probably due to its being inherently a 'weak' vaccine, and if failures are to be largely eliminated, a fully potent vaccine, more potent than would be considered adequate for primary vaccination, is required in re-vaccination. It is shown subsequently that in a small group of children, with the use of potent vaccine lymph in re-vaccination, no 'failures' were recorded even when the re-vaccinations were done only six months after successful primary vaccination. The possibility that the presence of the Duran-Reynals factor in calf lymph which contains, besides the vaccinia virus, living and dead bacteria, and its absence in bacteria-free membrane lymph may contribute to this difference in the ability to 'take' in partially immune individuals has also to be considered.

'Immune reactions' were recorded in 53.1 per cent of children vaccinated and re-vaccinated with membrane lymph and 41.3 per cent of children vaccinated and re-vaccinated with calf lymph. The reason for the higher percentage of the occurrence of the 'reaction of immunity' in children originally vaccinated with an admittedly weaker vaccine requires to be explained. That it bears relation to the use of a weaker lymph in re-vaccination is brought out by the very high proportion, viz., 93.8 per cent of these reactions recorded in group (iv), who were more adequately protected with calf lymph in 'primary' vaccination. Leake (*loc. cit.*) has observed that the use of insufficiently potent vaccine in re-vaccination results in a reaction similar to the 'reaction of immunity' in those who should give vaccinoids.

A comparison of the proportion of 'accelerated reactions' and frank 'takes' in the different groups also brings out the difference in the potency of the two types of lymph. The highest proportion (78.8 per cent) of these successful reactions occur in group (iii), children who were primarily vaccinated with membrane lymph and re-vaccinated with calf lymph; 57.6 per cent were frank 'takes' and 21.2 per cent modified 'takes' or accelerated reaction. In group (i) children originally vaccinated with membrane lymph, who may be assumed to be comparable in their vaccinal state to this group, when re-vaccination was done with membrane lymph the percentage of these successful reactions was only 14.5 of which 3.4 were frank 'takes' and 11.1 modified 'takes'. The percentage of successful reactions in the group originally vaccinated with calf lymph and re-vaccinated with the same lymph was 55.2, of which 20.9 were frank 'takes' and 34.3 modified 'takes'. Compared with the group primarily vaccinated with membrane lymph, these show a slightly higher residual immunity at the end of 4 years. The lowest percentage of successful reactions was in the last group of children who were originally vaccinated

with calf lymph and in whom re-vaccination was done with membrane lymph, being only 6.3. These figures again bring out the advantage of carrying out re-vaccination with a fully potent lymph. If the general view is accepted that the occurrence of these successful reactions following re-vaccination denotes susceptibility of the individual at the time of the re-vaccination, the fairly high percentage of these reactions obtained in groups (ii) and (iii) would indicate how rapidly the immunity afforded by primary vaccination in infancy can be lost, at any rate to vaccinia virus, and point to the desirability of carrying out re-vaccinations when the child enters school. The results of re-vaccination in a group of 42 children with a potent calf lymph six months after 'primary' vaccination are given in Table II :—

TABLE II.

Results of re-vaccination with calf lymph six months after primary vaccination.

Primary vaccination :—	Membrane.	Calf.
Re-vaccination :—	Calf.	Calf.
'Failure'	0	0
'Immune reaction' ...	11	11
'Accelerated reaction' ..	9	9
'Takes'	2	0
TOTALS ..	22	20

It is seen that with the use of a fully potent vaccine for re-vaccination, there are no failures, even when the re-vaccinations were performed within as short a period as 6 months after 'primary' vaccination. The high proportion of 'accelerated reaction' and 'takes' at such a short period is remarkable. 'Immune reactions' were recorded in a little over 50 per cent of these children.

In the course of a study of 2,000 consecutive cases of smallpox in the city of Madras,* during the period 1944-46, an analysis was made of the vaccinal state

* To be published separately.

of the patients at the time of the attack in relation to the severity of the infection and outcome of the disease. A comparison of the data relating to the case mortality rates in two groups (a) those who had been primarily vaccinated in childhood and (b) those who in addition to 'primary' vaccination in childhood were re-vaccinated but recorded as 'failures' is presented in Table III:—

TABLE III.

Mortality rates in cases of smallpox vaccinated once in infancy and in cases in whom re-vaccinations prior to attack were recorded as 'failures'.

Age groups.	PRIMARY VACCINA- TIONS ONLY.		RE-VACCINATION : 'FAILURES'.	
	Attacks.	Deaths.	Attacks.	Deaths.
0— 5 ...	33	8	1	0
6—10 ...	45	6	18	0
11—20 ...	218	18	138	2
21—30 ...	274	27	182	9
31—40 ...	66	15	41	1
41 and over ...	35	4	25	3
TOTALS ...	671	78 (11·6 per cent).	405	15 (3·7 per cent).

It must be stated that the results of re-vaccination were not observed earlier than a week after re-vaccination and that the 2nd group includes a large percentage in whom an 'immune reaction' would have been recorded had they been observed earlier. Both vaccinations and re-vaccinations in this group were performed with the routine calf lymph issued from the Institute.

These results show that those in whom a re-vaccination was done though without eliciting either an 'accelerated reaction' or a frank 'take' had higher resistance to withstand the attack than those in whom no re-vaccination was done.

DISCUSSION.

The observations recorded above show that with the use of a fully potent vaccine for re-vaccination, the absence of any reaction following re-vaccination is very uncommon and that a fully potent vaccine, more potent than would be considered adequate for primary vaccination, is required in re-vaccination. The

experience presented in Table III confirms this view that even at the end of 6 months, after successful primary vaccination, there was no case of 'failure'. Appreciably high percentage of 'accelerated reaction' and frank 'takes' are obtainable even within as short a period as six months.

The reaction following re-vaccination should, as pointed out by Edsall (1947), be considered in the relation to the level of immunity in the host and the potency of the virus preparation used. In his view, the 'immune reaction' would result when the immunity level in the host is sufficient to suppress the particular virus infection in question before it gets fairly started, and the chance of producing such a reaction would be favoured not only by a very high level of immunity in the host but also by a relatively low degree of infectivity of the virus. If the assumption is made that at the end of 4 years the level of residual immunity following primary vaccination with a more potent lymph is higher than the level of immunity following vaccination with the weaker membrane lymph, the figures for 'immune reaction' in the two groups would agree with his views.

Another difficulty in the interpretation of the significance of the 'immunity reaction' in re-vaccination arises from the fact that this reaction may be closely simulated by a reaction due to sensitivity which is apparently elicited by the constituents of the vaccinia virus, living or dead. Broom (1947) has drawn attention to the occurrence of a reaction indistinguishable from 'immune reaction' in approximately 10 per cent of persons tested by him in whom a simultaneous vaccination was done with a killed virus. Scott and Warin (1947) have shown that the killed vaccine elicits a similar reaction and that this is due to allergy resulting from previous sensitization of vaccination and can be elicited in equal measure with either heat-killed or viable virus. In a small series of cases where simultaneous vaccination was performed using a membrane virus, heat-killed membrane virus and uninoculated chick-embryo membrane supplied from the Institute, McPherson (1947—personal communication) has shown that the reactions elicited by the live virus and heat-killed membrane virus were similar and that the membrane element itself did not play any part. All workers of experience in this field are agreed that it is difficult to lay down any objective standard to differentiate the reaction of immunity from a reaction due to sensitivity to vaccinia-virus protein.

However, Broom (*loc. cit.*) has recorded 'immune reactions' in 119 out of 1,227 persons re-vaccinated by him, by carrying out a simultaneous control vaccination with a heat-killed virus preparation and interpreting his results on the basis of the difference in the intensity of the reactions observed at the sites where the live and killed vaccines were applied and on a knowledge based on the history of previous vaccination and the evidence of the scar left as a result thereof. Edsall (*loc. cit.*) re-arranging Broom's data to show the percentage of individuals manifesting the different types of response to re-vaccination has demonstrated that the percentage of susceptibles ('primary' and 'accelerated reactions') correlates directly with the interval since last vaccination and the percentage of 'immune reaction' shows an inverse correlation. If, therefore, a potent virus preparation is used along with a suitable technique, it is felt that it should be possible to recognize a reaction of immunity (*sic*) which could be taken as a manifestation of successful re-vaccination. It would, therefore, seem not to be entirely reasonable to classify all reactions other

than an 'accelerated reaction' and 'primary' vaccinia as unsuccessful. The case of 22 'immune reactions' presented in Table III in children who were re-vaccinated six months after 'primary' vaccination will be a point. The certificates of their original vaccination would remain valid for 3 years and the results of re-vaccination six months after successful primary vaccination would place them at a disadvantage. Such a position is obviously untenable. It is, therefore, our view that if re-vaccinations are performed with a fully potent vaccine, a technique adequate to ensure penetration of the host tissue applied and the results of the re-vaccination observed at suitable intervals after operation, 'immune reactions' recorded as such by experienced physicians would constitute evidence of a successful vaccination and imply that the person manifesting this reaction possesses a reasonable resistance to smallpox.

Our thanks are due to our colleagues in the King Institute and the personnel of the Department of Public Health, Government of Madras, and the Corporation of Madras, who have helped us in this study.

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TYPHUS GROUP OF FEVERS IN JUBBULPORE AREA.

BY

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AFTER the termination of the last war the Medical Directorate at G.H.Q. (India) decided to investigate the following selected areas of the country for the presence of typhus group of fevers and particularly their vectors: Kumaon hills in the outer Himalayas, Jubbulpore and Chindwara forest in the Central Province, Bangalore and Chedleth forest in the south and parts of the Rajputana desert. Out of these areas in Bhimtal, Ranikhet, Jubbulpore and Bangalore, typhus had occurred in the Army from a long time, but in the two forests its presence was detected when camps were started there during the last war.

The literature on the typhus fevers of India is fast accumulating and it will be more suitable to review it towards the end of these investigations, which by now include Kashmir also. The present paper is only a record of the investigations at Jubbulpore, which were carried out during the period of November 1946 to April 1947.

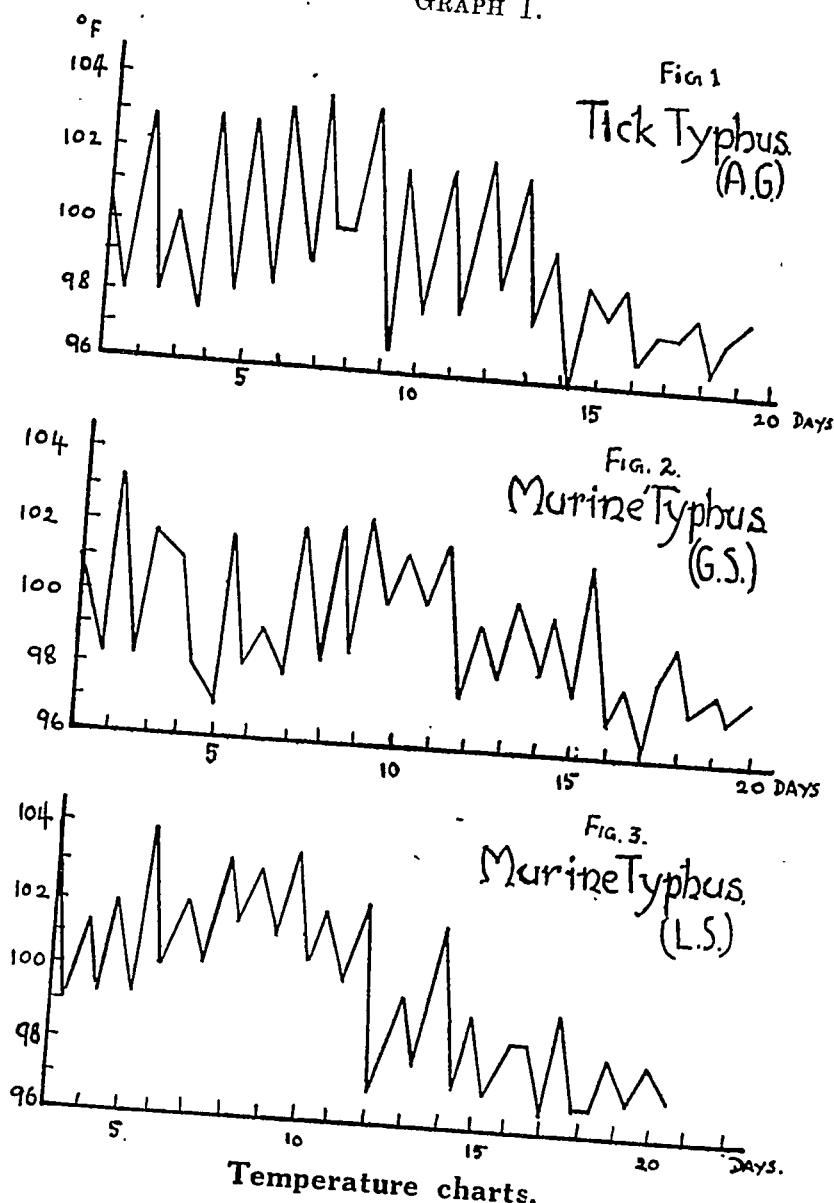
CLINICAL INVESTIGATION.

Murine typhus.—During the period of investigation, 11 cases occurred in the garrison at Jubbulpore. Ten cases had all the symptoms of a moderately severe attack of murine typhus. The following is an analysis of some of the particular symptoms: Superficial lymph glands enlarged in three cases, of these one had generalized lymphadenitis; in the second only the post-cervical; and in the third only sub-mandibular lymph glands were involved. Pain in the joints in one case; neuritis in one case; tympanitis in two cases and basal congestion of the lungs in one case. Rash appeared in 7 cases. The eleventh case was very mild with

Typhus Group of Fevers in Jubbulpore Area.

hardly any toxæmia and the fever lasted for 9 days only. All cases survived. The different types of temperature charts are shown in Graph 1:—

GRAPH 1.



Tick typhus.—In six months there was only one case of tick typhus (Abdul Gaffar, male, aged 22 years). Clinically, it could be called a mild case of Rocky Mountain spotted fever. The temperature chart (Graph 1, fig. 1) very closely resembled the one illustrated by Cox (1948) for spotted fever. The rash appeared on the 3rd day of the fever but did not become confluent and desquamation started during convalescence. There was a patch of consolidation below the left infra-clavicular

region which cleared by itself. The temperature came down by rapid lysis on the 17th day.

LABORATORY DIAGNOSIS.

Weil-Felix reaction.—Weil-Felix reaction was positive in 10 cases in a diagnostic titre (see Table I), but it did not help in differentiating between different typhus fevers. Seven cases had high OX2 agglutinins, while in three OX19 was high. In the eleventh case, in which fever had come down on the 9th day, the *Proteus* agglutinins were low. The case could not be followed, but its blood had been inoculated into a guinea-pig and the diagnosis was confirmed by positive complement-fixation test on the animal. The case of tick typhus developed a titre of 1:1,000 for *Proteus* OX2 only on the 22nd day of the illness.

Complement-fixation test.—These tests were carried out by the Base Typhus Research Laboratory at Poona, with murine, epidemic and Rocky Mountain spotted fever rickettsial antigens prepared by Lederle Laboratories Division. In 10 cases the test was positive with murine antigen only and negative with others. The serum of the tick typhus case was positive with spotted fever antigen only in a titre of 1:40++++. The differential diagnosis in this case was mainly based on the result of this test. The results of these tests are given in Table I:—

TABLE I.

Weil-Felix and complement-fixation tests of murine typhus cases.

(Both the tests done with the same serum.)

Month of onset.	Case.	WEIL-FELIX TEST.			Complement-fixation test with murine antigen.*
		OX2.	OX19.	OXK.	
June 1946	... M. S.	1,000	125	50	40 (3+) - 16
August 1946	... Indershy	5,000	0	0	40 (4+) - 9
November 1946	... T. R.	500	0	25	20 (4+) - 18
November 1946	... D. I.	5,000	0	0	40 (4+) - 20
November 1946	... Astley	800	100	25	80 (3+) - 14
December 1946	... B. S.	800	100	50	80 (3+) - 28
January 1947	... L. S.	500	125	50	40 (4+) - 21
January 1947	... Jain	25	500	50	40 (4+) - 20
April 1947	... G. S.	200	1,200	50	30 (4+) - 17
April 1947	... B. R.	200	1,600	50	40 (4+) - 20

* Legend.—40 (3+) - 16 means 1:40++++ on the 16th day and so on.

Isolation of strains.—Strains were isolated by inoculating 5 c.c. whole blood of the patient intraperitoneally into a guinea-pig. Three strains were isolated from murine typhus cases and one from the tick typhus case (see Table IV).

EPIDEMIOLOGY.

General.—Jubbulpore, Seoni and Chindwara are in the Deccan trap where the heights are from 1,000 to 3,000 ft. above sea-level. The total annual rainfall is 57 inches. The dry and hot months are March to May. The monsoons strike in June and 93 per cent of the precipitation is from June to October. The months of November to February are cool with moderately high humidity ranging from 63 to 76 per cent. The deciduous monsoon forests of Seoni and Chindwara are in the Satpura Range and are rich in wild life, rodents and insectivores.

Incidence of typhus.—The total cases of murine typhus in Jubbulpore Garrison from 1939 to 1946 were 97, out of these 77 per cent were in the winter months of November to February. Scrub typhus cases during these eight years were 22, chiefly in the monsoon period. Tick typhus appears to be a rare disease in spite of the abundance of ticks.

Insect vectors : Rat-fleas.—Jubbulpore town maintains a high rat population with a high flea index. Thirty rats were trapped in November, 253 in February and 154 in April. The total fleas recovered from these rats were 4,564. The flea index in these months was 4.9, 14.0 and 5.5 respectively. From this collection 135 fleas picked at random were identified; of these 74 per cent were *X. braziliensis* and 23 per cent *X. cheopis*, four fleas remained unidentified. In the Cantonment area 15 rats were trapped in the month of November and only five had a few fleas on them. No fleas were found on the forest rats.

Ticks.—As vectors of typhus our direct concern are ticks present on low vegetation for those are the ones that are likely to attack man. Therefore, greater attention was directed to their scatter on the ground rather than to their distribution on animals.

A white woollen cloth 5 ft. by 2 ft. attached to a long pole was trailed on the ground and over low vegetation. To get comparable results for different areas the flag was trailed for a known period every time and the figures reduced to a 30-minute trailing period. Thus, large areas were covered and representative random samples of ticks were collected. The method was particularly useful for surveys of forest areas where the only other alternative of examining wild animals was not practicable. Sometimes a large number of seed ticks was found on a space of one to two inches on the flag, indicating the presence of small clusters on the vegetation. These clusters were the greatest drawback in a quantitative comparative survey, but the error was reduced by covering large areas. Some of the observations made in these surveys were as follows :—

In Seoni and Chindwara area the villages were scattered in forest clearings. The ticks were mainly localized in the cattle-sheds, very thinly spread out over the fields but the density again increased in the surrounding forest which was used for cattle-grazing. In a wild-game forest the ticks were more or less uniformly spread out over the main part, with a gradual decrease towards the periphery.

Taking roughly equal areas there were more ticks in a wild-game part of the forest than in the part used for grazing.

The banks of forest lakes, where game tracks leading to them could be observed, had far less ticks on the grass near the water-edge than on the dry grass and bushes a few yards away from the water-edge. The bushes in a more or less marshy ground were not preferred by ticks. In the same area less ticks were caught immediately after the rain than before it. A dry day was more suitable for a survey than a wet one.

At Khamaria near Jubbulpore, a large number of ticks was collected from vegetation in January. In the beginning of May, in the same place, no ticks were found in one attempt and second time a few were collected from one or two bushes at one spot. In Rukhar forest near Seoni in the beginning of March as many as 440 ticks were collected in one hour, but in May only 5 ticks were found in one hour in the same area. In Chanda forest in May, there was no catch in one hour. This forest was not surveyed earlier, but according to the staff of the Forest Department, it is heavily infested during the rains and after. It appears that the tick density in these forests increases during the rains and is maintained throughout the mild winter but is rapidly lowered in the hot and dry summer. There is no evidence of winter hibernation of ticks in these parts.

Identifications of a random sample of 235 ticks are given in Table II. *Boophilus australis* was the most common tick in game forests, fields, as well as on domestic animals.

TABLE II.

Genera of ticks collected from different sources.

Source.	Total.	<i>Boophilus.</i>	<i>Hyalomma.</i>	<i>Rhipicephalus.</i>	<i>Hæmaphysalis.</i>
Animals ...	177	150	15	9	3
Fields ...	25	19	3	3	0
Forests ...	33	26	6	1	0

Mites.—Chindwara forest was surveyed for mites and their hosts in November and December, and Jubbulpore suburbs in January. Thirty-six traps were set every evening, with the same bait and by the same persons, covering roughly equal areas.

In Chindwara, over the greater part of the forest, there were more rats than field mice, except in one area near Singhori. Here the field mice (*Mus platythrix* Bennet) predominated rats, the ratio being 4.3 : 1, and most of the catches were in bushes

near streams or ponds (see Table III). Eighty per cent rats, 86 per cent field mice and 63 per cent shrews were infested with larval mites. Three *Tatera indica* caught had no mites on them. There were more mites on catches in thick grass and stream banks than in the fields. Fair-sized clusters were present in the ears and considering the off season the infestation was considered heavy. *T. deliensis* Walch was the predominant species and formed 70 per cent of the total. The rest of the species have not yet been identified.

In Jubbulpore suburbs, 27 rats out of 93, i.e. 30 per cent, and 2 out of 6 shrews, were infested with mites. There were no *T. deliensis* on shrews, while on rats it was not the predominant species.

TABLE III.

Showing the relative density of rats, field mice and mites in different vegetation types.

Vegetation type.	Rats.	Field mice.	Mites.
Cultivated fields	3·1	1·3	4·5
Thick grass	4·3	0	7·0
Banks of streams and ponds ...	1·0	4·0	7·0

Legend.—The rodent calculation was average per night catch. The figures for mites were arbitrary counts of 1 to 3, depending on the size of the cluster.

ISOLATION OF STRAINS FROM INSECT VECTORS AND RODENTS.

Rat-fleas.—A total of 4,002 fleas from 257 rats trapped in Jubbulpore was examined in 8 batches. Two strains of rickettsia were isolated. Every batch was a mixed pool of *X. cheopis* and *X. braziliensis*.

Ticks.—Twenty-three batches of ticks were examined and one strain of murine rickettsia ('O' strain) was isolated from a batch of *Boophylus australis*. This strain is described separately at the end. No strain of tick typhus rickettsia was isolated.

Mites.—No infection was discovered in 20 batches of mixed pools of *T. deliensis* and other species.

Rats.—Twenty rats from Chindwara were examined for *R. orientalis* infection and one strain was recovered. No rats were examined for murine infection.

Field mice.—Six mice (*Mus platythrix* Bennet) were examined and one strain of *R. orientalis* was recovered.

Three shrews and two *Tatera indica* were also examined for *R. orientalis* but showed no evidence of infection.

TABLE IV.

Showing the source, number and type of strains isolated; also the results of complement-fixation tests with guinea-pig sera on the 40th day after inoculation.

Source.	Number.	Strain.	Type.	COMPLEMENT-FIXATION TEST.*		
				ER.	MR.	RMSF.
Typhus cases ...	4	A	Tick typhus	0	0	30 (4+)
		B	Murine	0	20 (3+)	0
		C	Murine	0	80 (4+)	0
		D	Murine	0	80 (4+)	0
Fleas ...	2	J	Murine	0	20 (2+)	0
		N	Murine	0	40 (2+)	0
Ticks ...	1	O	Murine	0	40 (4+)	0
Rats ...	1	...	<i>R. orientalis</i>
Field mice ...	1	...	<i>R. orientalis</i>

* Legend.—ER, MR and RMSF are respectively epidemic, murine and Rocky Mountain spotted fever antigens.

COURSE OF INFECTION IN EXPERIMENTAL ANIMALS.

Murine typhus infection in guinea-pigs.

General reactions.—The reactions were the same with strains from typhus cases as well as fleas and were typical of these rickettsia. The incubation period varied from 3 to 19 days. The temperature in most of the animals showed two peaks: the first, short and sharp, preceded the main rise by 2 to 9 days; others had only one rise lasting from 5 to 9 days. This variation was not characteristic of any particular strain. The scrotal reaction was a 'tunica reaction' and followed the temperature curve in its intensity and duration. It was present in the majority of animals, but was not produced consistently by any strain. Adhesions in the scrotal sac were seen in most of the animals, but a sticky exudate was present in all of them. Rarely hæmorrhages were present under the muscle-sheath of thigh, pectoral or rectus muscle, and tiny hæmorrhagic spots on the bladder-wall.

Appearance of the rickettsia.—The smears were stained with Machiavello stain. Bacillary type of rickettsia was rarely seen, largely red inclusion bodies were present in varying sizes and numbers in the cytoplasm of the cells. Some of the cells had a distended appearance, the cytoplasm was vacuolated and the vacuole

filled with large pink granular bodies which may be micro-colonies of rickettsia. In a few animals only blue inclusion bodies were seen, similar to those mentioned by Sacha (1946) in epidemic typhus.

Serology.—There was seldom any rise of *Proteus* agglutinins in guinea-pigs, but the complement-fixation test was always positive, with murine antigen only, in all animals that reacted with fever. The results are given in Table IV.

Two strains, D and N, one from a patient and the other from fleas, were successfully maintained in white rats for over two years which is an indirect evidence that they were murine.

Tick typhus infection in guinea-pigs.

The first guinea-pig was inoculated with 5 c.c. blood of the patient (Abdul Gaffar), taken on the eighth day of the fever. The incubation period in the first three animals was 19, 15 and 18 days, respectively. The general and tunica reactions were of the same nature as in murine infection, the only difference being the marked redness of the foot-pads and later desquamation, but no necrosis. Rickettsia in the form of red inclusion bodies as well as bacillary type were seen in tunica and peritoneal smears. The strain, however, gradually died out in guinea-pigs. The complement-fixation test on the serum of an animal in the second pass, on the 40th day, was positive with spotted fever antigen in a titre of 1:30++++ and negative with epidemic and murine antigens.

Scrub typhus infection in white mice.

The mice showed all the signs of *R. orientalis* infection and typical rickettsia in the peritoneal smears, both with the rat and the field mice strain. Blue inclusion bodies (Giemsa stain) were seen in the peritoneal cells in the first animals in both the strains. The strains died out in the 4th passage, and although blind passes were carried out for eleven generations, they could not be revived.

Natural infection of murine typhus in ticks.

Ticks (*Boophilus australis*) were collected from bushes near a Military camp close to the village of Khamaria about 10 miles north of Jubbulpore and inoculated into a guinea-pig.

Reaction in guinea-pigs.—The incubation period in the first animal was 10 days and in the second pass two and four days. Later, it was passed for three successive generations through white rats and then brought back into a guinea-pig. The incubation period in this animal was fifteen days, but six subsequent passes in guinea-pigs again reduced it to three or four days.

The tunica reaction lasting for varying periods of 3 to 6 days was present from the very beginning and appeared in all the male animals inoculated. The temperature, post-mortem appearances and morphology of the rickettsia were similar to other murine strains.

Out of the two animals in the second pass one was bled on the 11th day, its serum agglutinated OX19 1:400. The Weil-Felix reaction in all the other animals

inoculated with this strain was negative. The positive reaction in one animal was apparently an exception due to animal variation and not due to any particular property of the strain. The other in the second pass was bled on the 12th day when its complement-fixation test was negative, but on the 27th day it was positive 1:40++++ with murine antigen and negative with spotted fever antigen.

Reactions in a monkey.—A female monkey, *M. radiatus*, was given a subcutaneous injection of the virus from a guinea-pig. By the third day a hard and indurated lump developed at the site of inoculation which gradually subsided and disappeared on the 10th day. The fever started on the 13th day and lasted for 5 days.

The Weil-Felix reaction became positive on the 15th day in a titre of OX19 1:160, OX2 1:20 and OXK negative. On the 22nd day the OX19 titre came down to 1:80, where it stayed for four weeks before it fell below 1:20. The complement-fixation test was positive for the first time on the 22nd day in a titre of 1:20++ with murine antigen; the maximum titre of 1:160++ was reached on the 57th day. The blood was still positive on the 102nd day in a titre of 1:10++. Throughout this period the serum remained negative for Rocky Mountain spotted fever antigen.

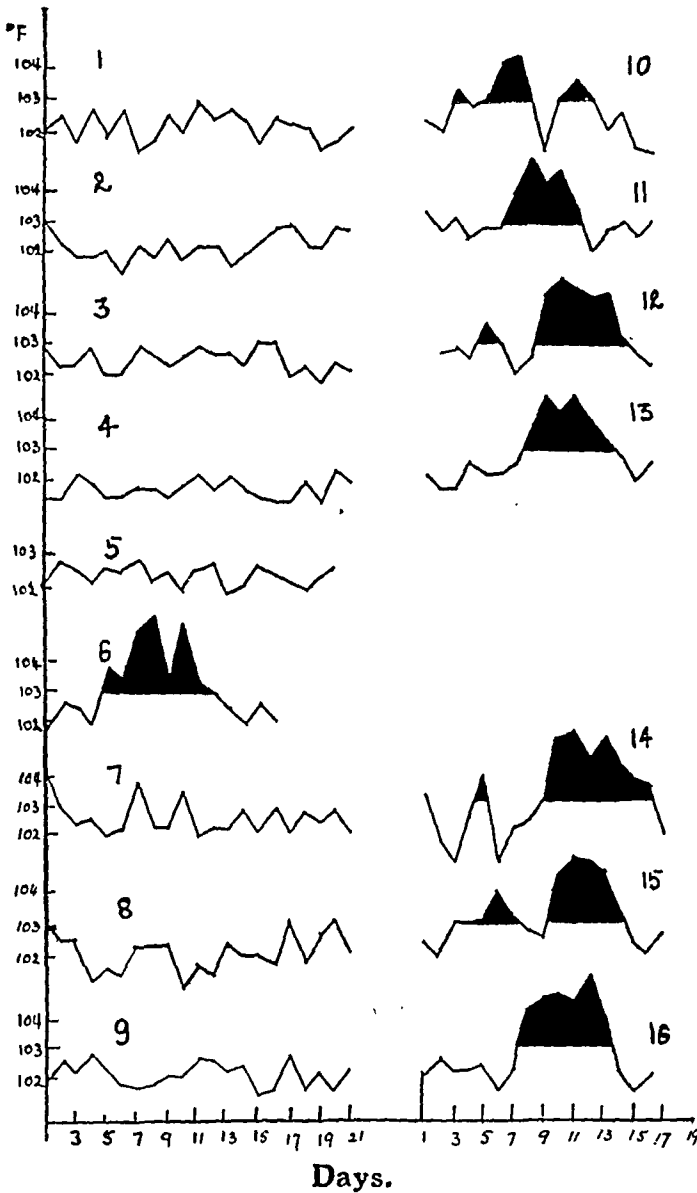
Immunity tests (see Graph 2).—Two guinea-pigs immunized with 'N' flea strain and two immunized with this strain gave complete cross-protection. One animal recovered from an infection of a murine strain from a patient in Jubbulpore was also completely protected; while another recovered from tick typhus infection from the patient strain (Abdul Gaffar) did not show any protection. Four controls, two each with the flea strain and this strain, reacted normally.

Three guinea-pigs were immunized with three weekly subcutaneous 1-c.c. doses of combined epidemic and murine vaccine of Connaught Laboratories. One animal was female and two males. The challenge dose with this strain was given intraperitoneally ten days after the last dose of vaccine. The female showed no reaction; in one male the temperature touched 103.0°F. on two days only but the tunica reaction did not develop, in the second male the temperature touched 103.5°F. and 103.3°F. on two days but the tunica reaction developed and the testes remained adherent for 5 days. Three controls, one female and two males, which received their challenge dose from the same material, gave a full reaction.

One may conclude from the above that with the local murine strains there was complete protection; while of the vaccinated animals one showed complete protection and the two only a partial protection.

Lewthwaite *et al.* (1936) failed to infect *D. andersoni* and *R. sanguineus* ticks by feeding on infected animals. On the other hand, Zinsser and Casteneda (1931) were able to infect *O. octocanther nitens* and *Amblyoma* species by rectal injection after which the rickettsia remained alive in these ticks for at least 12 days. This is the first time that ticks were found infected in nature with murine rickettsia.

GRAPH 2.



GRAPH 2.—Showing protection tests on guinea-pigs with murine strain isolated from ticks and other strains.

Guinea-pig Nos. 1 and 2.—Immunized with murine strain from fleas.

” ” 3 and 4.—Immunized with murine strain from ticks.

” No. 5.—Immunized with murine strain from patient.

” 6.—Immunized with tick typhus strain from patient.

” Nos. 7, 8 and 9.—Protected with combined epidemic and murine vaccine.

” ” 1, 2 and 5 to 9.—Given a challenge dose of murine strain from ticks.

” ” 3 and 4.—Given a challenge dose of murine strain from fleas.

” 10 and 11.—Controls of murine strain from fleas.

” ” 12 to 16.—Controls of murine strain from ticks.

PROTEUS agglutinins in animals.

In a search for animal reservoir of tick typhus the sera of 51 sheep, 58 goats and 26 dogs were examined for the presence of *Proteus agglutinins*. The sera of 2 sheep, 6 goats and 8 dogs agglutinated OX19 or OX2 in a dilution of 1:160 or over. The complement-fixation test with murine and spotted fever antigens on two of these goats and one dog gave negative results. It is considered that Weil-Felix reaction is unreliable for a study of this nature.

DISCUSSION.

Murine typhus.—Cross-protection tests between strains isolated from patients and fleas were not carried out. There was, however, enough evidence that the disease was present in the area. The season was November to February when the climate was cool and humid and well suited to the fleas. In the Cantonment the cases were rather few, also the fleas were much less than in the town. On the other hand, in the town where the flea index was high and infection was present in the fleas, there was no report of any case. This can only be attributed to missed diagnosis, due to unawareness of the presence of the disease and lack of laboratory facilities.

The possibility of ticks conveying murine typhus becomes an open question, as they were found infected in nature in this area, although their vectorship was not proved. One case of murine typhus (Lieut.-Colonel Indersby) (see Table II), resident of the area where these ticks were collected, gave a definite history of tick-bite eight days before the fever. This, of course, may be regarded as an indirect evidence or a coincidence.

Tick typhus.—The only case of tick typhus gave a positive complement-fixation test with Rocky Mountain spotted fever antigen in a diagnostic titre. The Base Typhus Research Laboratory at Poona had received other sera from many parts of India which gave fixation with this antigen, but this is rather insufficient evidence to say that these cases were similar to Rocky Mountain spotted fever. The disease in India is probably mild as compared to typical spotted fever and produces a mild infection in guinea-pigs, also the strains are difficult to maintain in this animal. The positive complement-fixation with spotted fever antigen may only be a cross-fixation as obtained in Boutonneuse fever. Therefore, so long as the complement-fixation tests are not carried out with highly purified washed antigens of spotted fever along with a similar antigen prepared from the local strain, and as long as strains in India are not compared with strains of spotted fever, the disease here may be provisionally called 'tick typhus'.

Scrub typhus.—The scrub typhus strains were isolated in the winter months and died out in white mice. This was contrary to the experience with strains isolated in Imphal and Kumaon hills during the scrub typhus season. It is well known that some strains do not show any evidence of infection in the 4th to 7th passages but are revived in later passages. It is possible that towards the end of the season, when the infection in the rodents is dying out, the rickettsia are partially attenuated and may not revive.

Blue inclusion bodies were present in peritoneal smears in white mice that received material from nature. Previously, Kalra (1947) had observed similar

bodies but staining red, which from the evidence available were believed to represent a phase in development cycle of the rickettsia during the stage of adaptation to a new host. Whether these inclusions were of a similar nature was not possible to say. Further observations on the points raised above are needed.

SUMMARY.

1. The different typhus fevers of Jubbulpore area, their clinical symptoms, laboratory diagnosis, epidemiology, and animal reactions with the strains isolated are described.

2. A strain of murine typhus rickettsia was isolated from ticks, *Boophilus australis*, collected from bushes in the suburbs of Jubbulpore.

Major J. H. Stirrat of the Base Typhus Research Laboratory, Poona, carried out all the complement-fixation tests. Captain S. P. Varma co-operated in the clinical work. The mites were identified by Dr. Charles D. Radford and the rodents by J. L. Harrison, both of the British Museum. Lieut.-Colonel M. L. Ahuja read the manuscript of this paper and made many useful comments, particularly in the arrangement of the text. We are grateful to all of them for their help.

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TICK-BORNE RELAPSING FEVER IN KASHMIR.

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RELAPSING FEVER had remained unrecognized in Kashmir before February 1948, although on inquiries indirect evidence was collected of its previous presence. There was a belief in some parts that the bite of *Ornithodoros* ticks causes malaria. Some local doctors used to resort to intravenous arsenic therapy with good results in cases of clinical malaria intractable to quinine. The widespread occurrence of the disease and the vector also point to old endemicity. In all likelihood it had been confused with malaria due to resemblance of symptoms, no case fatality, and particularly lack of laboratory aid in diagnosis. Moreover, ticks of *Ornithodoros* species had not been recorded from these parts.

In February 1948 cases of relapsing fever were first reported by Lieut.-Colonel Lobo in troops stationed in Jammu province. In March 1948, five cases were microscopically diagnosed in personnel stationed at Drugmulla in Kashmir valley. The patients had no lice on their body but all had slept in a particular room of a building. Two *Ornithodoros* ticks were found on searching these rooms. These ticks, when fed on a guinea-pig, infected it with *spirochaetes* morphologically resembling those seen in the blood of cases. Starting with this evidence, a survey of the greater part of the State, for different species of *Ornithodoros* ticks, was carried out. Observations on bionomics of ticks and epidemiology of the disease were made for one year. The disease and its therapy were studied by Captain S. K. Chopra and is reported separately.

SPECIES AND THEIR BIONOMICS.

Three species of *O. crossi*, *O. lahorensis* and *A. persicus* were found in Jammu and Kashmir.

O. crossi.

Habitat and hosts.—These ticks were universally distributed over Jammu and Kashmir. They had a definite association with cattle, sheep or goats and were found in any house or hut where these animals were housed or had been quartered at any time. They were also collected from the out-houses of dak bungalows and houses, where the presence of faecal remains showed that sheep or goats had been kept there at some time. They were never found in horse or mule stables.

In summer months the largest numbers were present in the floor dust, particularly near the walls, dark corners, loose dust around the wooden roof-props and rat-holes; lesser numbers were found behind the mud plaster on the walls. The density in the walls decreased from floor upwards, and very few were found above heights of 4 feet. The next common hiding place was cracks and dust on the window-sills. The ceilings were not examined. In winter the situation was reversed and more took shelter in the walls behind the mud plaster than in the floor dust. In 'gujjar' huts where the ventilation was poor and the room temperature, due to animal heat and a constant fire, ranged from 21°C. to 25°C. even when the outside temperature was 2°C. or below, the ticks were seen roaming on the floor near the cattle. There was no evidence of winter hibernation in such places for 60 to 70 per cent were freshly engorged in all weekly collections from December to end of February.

Throughout the year one never saw them on animals, but indirect evidence was obtained of their parasitizing cattle, sheep and goats. Their frequent presence in rat-holes suggested feeding on this animal also. The association of *O. tholozani* with rats in Cyprus has been suggested by Gambles and Coghill (1948). The dried blood of 29 freshly engorged ticks collected from houses in Kashmir valley was examined by Lieut.-Colonel S. D. S. Greval. He reported that 6 gave positive precipitin tests for sheep or goats, 9 for buffalo or cow and 12 for both the groups. Two bloods were negative for these animals and also did not react with anti-human or anti-fowl serum. These two could have only fed on rats. In the laboratory they readily accepted feeds on a white rat, guinea-pig, rabbit, monkey, goat, sheep and man.

Wheeler (1938) had noted that in nature ticks sometimes suck blood from other ticks. To confirm this a freshly engorged larvæ was kept with an unfed one in the same tube. In a few minutes the latter started sucking the blood of the engorged one who died afterwards. Similar parasitism was observed twice again with nymphs. In one case two first instar nymphs were trying to suck blood from a full-grown engorged nymph.

Feeding habits.—A starved tick seldom wandered on the animal but settled down to bite wherever it was dropped, this was particularly so with larvæ and first instar nymphs. The feeding time varied with different ticks and with the same tick on different occasions. The range of time taken to finish a feed was from 11 minutes to 10 hours, but over 50 per cent always finished in less than one hour irrespective of the host (see Table I). The following times in minutes were taken by two ticks during different feeds on guinea-pigs :—

First tick—11, 21, 41, 119, 321, 12 and 25 minutes.

Second tick—18, 26, 24, 21 and 105 minutes.

TABLE I.

Showing percentage of ticks (nymphs and adults of *O. crossi*) finishing a feed at different times on different animals.

Animal.	Total ticks.	Time in hours :										
		$\frac{1}{2}$	1	2	3	4	5	6	7	8	9	10
Guinea-pig ...	320	31.8	31.0	21.0	6.8	2.5	0	1.0	0	0	4.4	1.5
Rat ...	120	52.5	15.8	20.0	10.0	1.7
Goat ...	100	38	28	24	9	1
Rabbit ...	100	30	34	12	12	10	2
Monkey ...	63	79.4	3.1	1.6	9.5	1.6	4.8

Bite marks.—In one trial on one of us, there was no sensation or hurt when the tick started biting or during the time it remained sticking. When it detached itself the mark left behind was dusky red in colour with a dark centre 3 mm. in size. Next day the colour was darker red, a small papule appeared in the centre, the base was indurated and there was mild induration off and on. From the third day the colour began to fade and the induration was on the decrease. On the fifth day there was a light-dark circular mark, which gradually faded and completely disappeared on the 10th day. The presence of bite marks on a patient will naturally depend on the incubation period, which is variable and can be more than 10 days.

Effect of light and temperature.—In general all stages of ticks had the tendency to move away from the direction of light, except a starved tick which sometimes wandered facing the light. To study the effect of temperature the experiments were carried on last instar nymphs and adults of *O. crossi* a week after feeding. Fine dust was spread on the floor to imitate the natural habitat. The room temperature was controlled with a 'Bukhari'. The tick was left on the floor, its track was traced with a pencil, a thread was taken over the track and measured. In the first experiment two different batches of 5 ticks each were tried at 10°C. and 30°C. The average distance covered by a tick in 10 minutes was 35 inches at 10°C. and 99 inches at 30°C. As the speed of different ticks varied, in the next experiment the movements of the same 6 ticks were recorded at different temperatures. It was again confirmed that the speed increased with the rise in temperature (see Table III).

TABLE III.

Average distance in inches covered by a tick in
10 minutes.

Distance.	Temperature in °C.				
	2	12	16	20	27
Original course ...	2.7	41.2	66.2	70.4	80.2
Straight distance ...	2.0	30.0	59.2	59.5	67.3

Next the speed of 16 ticks was measured, before and immediately after engorgement, at room temperature fluctuating between 20°C. and 22°C. The average distance in 10 minutes covered by a starved tick was 74.5 inches and after a feed 54 inches. The course followed by a tick was not very zig-zag and on an average the loss from a straight distance was only 13 per cent.

Six ticks were kept in a bare Petri-dish and another five in a dish with its surface thickly covered with dust and grass. Both were exposed to direct sun when the temperature was 42°C. The ticks first became agitated and then motionless. In the bare dish all died in 3 minutes, in the second dish all were motionless in 15 to 20 minutes, but two revived after an hour in shade.

To know whether a tick will bite at low temperatures, 7 starved ticks were left outside in a test-tube for some minutes when the atmospheric temperature was between 3.5°C. to 4°C. and then dropped on a guinea-pig. Three were stuck in 3 minutes, 2 after nine minutes and 2 refused a feed. For control 7 ticks from the same batch were put on a guinea-pig inside the laboratory, where the temperature was 20°C. Six were stuck in 3 minutes and one refused feed. It shows that although the movements of a tick will be slow at very low winter temperatures of Kashmir, but if it reaches a host it will bite, if hungry.

O. lahorensis.

This species were as widely distributed as *O. crossi* and their summer habitat was also the same. Sheep were the selective winter hosts and all examined were found infested with *O. lahorensis*, from December to end of February, i.e. the time when they are sheared. Only once they were seen on a cow kept in the same room with sheep, but they were never seen on a goat. All the ticks collected from sheep were in different nymphal stages. The adults and engorged last instar nymphs were once found on the walls of a sheep-pen.

It did not appear to attack man readily for out of many attempts to make them feed on us only one succeeded. Amongst the laboratory animals it preferred rabbit to any other, probably because of the former's long soft fur.

Bite marks.—A recently moulted nymph attached itself on one of us on the lateral side of the finger near the base. It remained sticking for four hours, although after half an hour it appeared fully engorged. There was no sensation when it started to bite but later there was mild irritation, particularly during the first half hour. On detaching, it left behind a round dark-red mark, 5 mm. in size. During the night there was some local itching. Next day the red mark had faded but there was an inflammatory area around it with an indurated base. The entire longitudinal half of the finger on the side of tick-bite was benumbed with loss of sensation to light touch.

Second day a tiny pus-point appeared at the centre, numbness was still present, the inflammatory area and induration had increased and the morning body temperature was 99.2°F. Third day the numbness was less, swelling became localized, a vesicle 7 mm. in diameter appeared but the body temperature was normal. Fourth day there was no numbness and swelling. The vesicle gradually subsided on the 8th day, leaving behind a thick, firm, dark scab which separated off on the 25th day. Similar marks were seen on 6 persons in the whole of the year but none of them developed relapsing fever.

A. persicus.

Argus persicus was present in most of the fowl-houses examined. It refused feed on man or any laboratory animal except fowl.

Dispersal of ticks.—*O. crossi* was chiefly a domestic tick and its dispersal from one place to another appeared to be more by human agencies than through animal sources. The animal sheds in Kashmir are not cleaned throughout the winter but get a thorough cleansing on the approach of spring. All the rubbish removed is dumped in any open space in the village and the ticks in it gradually make their way back to the nearest houses. The other common observations reported by Army personnel were: (a) that they were carried in bedding; (b) that they were brought along with the dry hay bought from civilian houses for use as bedding in bunkers; and (c) that they reached a new building when a part of the material for its construction was taken from an old destroyed building. *O. lahorensis* gets transported even to distant places in winter on sheep.

INFECTION IN TICKS AND MODE OF INFECTION.

No *spirochaetes* were seen in the tissues of *O. lahorensis* and their bite did not convey infection to animals. *O. crossi* was not only found in all the places where cases of relapsing fever occurred, but *spirochaetes*, morphologically resembling those seen in the blood of cases, were also seen in their crushed tissues and their bite infected laboratory animals. The infectivity of an individual tick was irregular as already pointed out by Davis (1941), and the *spirochaetes* were not transmitted by every bite.

The mode of infection was considered to be directly through the bite and not by the contamination of the puncture made by the bite with the coxal fluid or faeces. Out of more than a 1,000 feeds, only in two cases the coxal fluid was discharged while the tick was still attached. Moreover, coxal secretions of 70 known infected ticks were examined microscopically and of 24 infected ticks were inoculated in to white rats and white mice with negative results. The faeces are ruled out, for the same were never seen discharged on animals during feeding.

STUDIES WITH *spirochaetes* FROM TICKS AND RELAPSING FEVER CASES.

The bloods of random samples from stock colonies of guinea-pigs, white rats and white mice were examined but no *spirochaetes* were seen in any. Nymphs and adults were used for transmission by feeding them on animals. To study human strains 1 c.c. to 2 c.c. of blood taken during the febrile stage was inoculated subcutaneously or intraperitoneally.

Guinea-pigs, white rats, white mice and monkeys (*M. radiatus*) were found susceptible to these strains, while rabbits and goats were refractory.

Infection in guinea-pigs.—Fourteen batches of *O. crossi* ticks were fed on different guinea-pigs, of these 8 became infected. One animal showed *spirochaetes* in its blood on the 9th day and later on 8 more occasions, but did not exhibit any fever. The rest had sharp pyrexial bouts after incubation periods of 5, 5, 7, 8, 8, 12 and 12 days. The febrile period lasts from 1 to 4 days. The relapses were only 2 or 3 at intervals of 3 to 5 days. The *spirochaetes* appeared in the blood either a day before the first rise or simultaneously with it and often persisted in the afebrile period.

Six animals were inoculated with the patient's blood. The incubation period was 3 to 8 days, the bouts of fever were 3 to 5 at intervals of 2 to 7 days.

The comparatively larger number of bouts than that produced by tick-bites was probably due to a heavy original infection conveyed through the patient's blood.

Normally, none of the guinea-pigs died of the infection but the virulence was enhanced on repeated passages carried over with infected blood. A strain from a case produced two relapses in the first animal, but the temperature never rose beyond 103.5°F. Its 2nd, 3rd and 4th passage guinea-pigs had more definite bouts of fever, while the 5th and 6th passage ones died of it. In the last two the *spirochaetes* were seen in smears from liver, spleen, lungs, kidney and bone-marrow of long bones.

Infection in white rats.—Six batches of *O. crossi* were fed on white rats. Five animals showed *spirochaetes* in their blood after 6, 6, 7, 9 and 13 days, respectively; but there was no sign of illness or rise in temperature. The bite of one tick was enough to convey the infection. Seven rats were inoculated with infected human bloods. The *spirochaetes* were first seen in their blood after 2, 3, 3, 3, 4, 5 and 13 days. A strain was recovered from the spleen of one rat a month after the disappearance of the *spirochaetes* from the blood. None of the rats died.

White mice were successfully infected with infected blood but did not die. Their body temperature was not recorded.

A female monkey (*M. radiatus*) was bitten by 36 ticks. The first rise in temperature occurred after 18 days and was followed by two relapses at intervals of 4 and 14 days.

A goat was inoculated with heavily infected blood from a relapsing fever case. During an observation period of one month, the temperature remained normal and no *spirochaetes* were seen in the daily blood smears.

Cross-protection tests in guinea-pigs.—The experiment was carried out with two strains, one from a patient and another from ticks. Three animals were first

inoculated with the tick strain and re-inoculated with the human strain; two were treated in the reverse order and one received the same human strain both times. The re-inoculation was done when the blood had been negative for one month after the first inoculation. There was only a slight evidence of partial immunity, for re-infection always occurred, though on the second occasion the *spirochaetes* disappeared from the blood rather earlier (see Table IV).

TABLE IV.

Showing the results of the cross-protection tests.

1st strain.	Bouts.	Days, blood positive.	2nd strain.	Bouts.	Days, blood positive.
Tick ...	3	8	Patient ...	2	4
Tick ...	1	10	Patient ...	1	4
Tick ...	3	15	Patient ...	2	10
Patient ...	3	8	Tick ...	3	4
Patient ...	2	15	Tick ...	1	5
Patient ...	2	3	Patient ...	0	2

The above experiments on guinea-pigs either prove lack of development of immunity or lack of specificity in the *spirochaete* species. On this subject, Nicolle and Anderson (1928) and Brumpt (1939) had disputed the concept of species unity, but Davis (1942) found an escape from the previous criteria and suggested that 'each species of *Ornithodoros* that is a relapsing-fever vector carries a *spirochaete* that is tick host-specific and this host-specific relation offers a more accurate approach to the differentiation of *spirochaetes* than any of the several criteria heretofore used'. If the idea elaborated by him be followed then the *spirochaetes* here might as well be considered a new species for *O. crossi* has not been recorded as a vector elsewhere.

Experiments with lice.—Lice collected from healthy persons were given two feeds on cases of relapsing fever when large numbers of *spirochaetes* were present in their blood. Afterwards they were fed twice a day on healthy persons. The mortality in lice was rather high and nearly one-third died in nine days or more. The crushed tissues of dead lice were examined each day and only once a *spirochaete* was seen in a louse that had died overnight after an infected feed. Two batches of lice were inoculated into white rats 9 and 11 days after the infected feed; 3 batches after 9, 11 and 17 days into guinea-pigs and one batch after 9 days

into a monkey. None of the animals became infected. Further experiments with longer incubation periods are being carried out.

RESERVOIR OF INFECTION.

The trans-ovarian transmission of *spirochaetes* in ticks was proved long ago by Dutton and Todd (1907) in Belgian Congo, hence the tick itself remains an important reservoir of infection.

The blood of 14 rats trapped in an endemic tick-infested area was examined microscopically and *spirochaetes*, morphologically resembling those seen in the blood of cases, were present in four of them. Therefore, it is quite possible, as suggested by Darling (1922) and Moursund (1942), that rats act as reservoir of infection. As previously stated, an attempt to infect a goat was a failure; moreover, 25 goats and 50 sheep were examined from an endemic area but no *spirochaetes* were seen in the blood of any. Hence these animals, although important hosts of ticks, probably do not act as reservoirs of infection.

In over 90 per cent of the relapsing fever cases the *spirochaetes* were abundantly present in the peripheral blood during the febrile period, therefore an untreated case could act as a temporary though periodic reservoir. This possibility which is also suggested by the work of Catanei (1923) was greater in Kashmir where the disease was not recognized and naturally the cases remained untreated and infective for a longer period. Consequently, an untreated case can not only infect more ticks but also spread the disease, if he travels during his illness.

To sum up, new lines of infection in ticks can be started by their sucking the blood of other infected ticks, or of infected rats, or of a case of relapsing fever.

EPIDEMIOLOGY AND DISCUSSION.

The disease was universally distributed over both the provinces of Jammu and Kashmir excluding Ladakh valley and cases occurred in rural areas as well as towns including Srinagar and Baramula. The incidence was related to temperature and was highest in the summer months of May to October (see Table V). In winter a bunker cannot be warmed and the locals, in order to conserve

TABLE V.

Showing maximum temperature, vapour pressure and monthly incidence of relapsing fever per 1,000 persons at risk from March 1948 to February 1949.

	1	2	3	4	5	6	7	8	9	10	11	12
Incidence ...	0.8	0.13	1.0	1.0	2.7	4.3	3.1	4.6	1.5	2.0	1.7	0.45
Temperature °C.	50.0	44.1	56.9	65.8	77.0	85.4	87.8	86.6	83.5	73.8	62.5	48.2
Vapour pressure	4.9	5.6	7.6	10.6	13.8	16.9	20.3	19.9	15.0	9.5	6.0	5.4

fuel, do not warm the room but keep their persons warm with a 'Kangri'. The low temperature of the room will reduce the activity of the tick and lower the incidence.

Two species of *Ornithodoros* were present but, for the reasons given below, it is considered that *O. crossi* was the only vector. It attacks man more readily than *O. lahorensis*. It was more abundant during the period of high incidence. Infected specimens were recovered from all the places where cases had occurred. Its bite mark was quite distinctive from that of *O. lahorensis* and resembled those seen on the patients.

Attempts to infect lice were unsuccessful and the high summer incidence was also against the louse being the transmitting agent.

SUMMARY.

1. Three species of *Ornithodoros* were found in Kashmir.
2. Certain aspects of the bionomics of two species—*O. crossi* and *O. lahorensis*—are dealt with.
3. *O. crossi* has been proved to be a vector of a form of relapsing fever in Kashmir.
4. Experiments conducted up to date indicate that the *spirochete* in question cannot be transmitted by lice.
5. Transmission is effected by the bite of the tick and not through the coxal fluid or faeces.
6. The cannibalistic tendency of ticks has been observed; this permits of a further means of dissemination of the infection from tick to tick.
7. The reactions of various laboratory and domestic animals to infection with the *spirochaetes* are discussed; some evidence that wild rats may act as reservoirs of infection has been obtained.
8. The widespread distribution of the disease was realized by its occurrence in the Army personnel stationed in various parts of Kashmir.

The authors owe thanks to Colonel S. Narain for help and suggestions throughout the work, to Lieut.-Colonel S. D. S. Greval, Serologist, Government of India, for carrying out precipitin tests on the blood of ticks, and to Dr. B. C. Basu, Entomologist, Indian Veterinary Research Institute, Muktesar for the identification of ticks.

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The ticks mentioned as *O. crossi* in the text were also sent to Dr. Glen. M. Kohls of Rocky Mountain Laboratory. He thinks that the nomenclature should be *O. tholozani* var. *crossi*.

TYPHUS FEVERS IN KASHMIR STATE.

Part I.

EPIDEMIC TYPHUS.

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THE earliest mention of typhus in the Medical Administrative Reports of Kashmir and Jammu State was in 1894, when a small outbreak appeared during spring near Bunji in Gilgit. The record gives no indication of this being an exceptional event and the disease had probably been present here from a long time. The next incidence was recorded in 1903, and from 1916 onwards the reports were more frequent. The biggest outbreak in recent times started in 1943 and was extended to 1944, resulting in 2,481 cases and 730 deaths. It spread over the whole of Baramula district and the northern part of Anant Nag district, including Srinagar. This period coincided with the immigration of over 5,000 Kazaks from Russian territory. They entered through Zogila pass, came down to Bandipor, Sopor and Baramula from where they were escorted to the western borders of the State. Quite a large number of them died of fever during this trek. It is possible that they were responsible for importing a fresh virus, for this epidemic started during their sojourn and was chiefly limited to the route followed by them. Coincidentally, during the same year Bayne-Jones (1948) records a similar outbreak of typhus in Iran started by the entrance of Polish refugees from Russia.

Besides Kashmir valley, outbreaks have frequently appeared in the hilly tracts of Jammu province and Ladakh valley. At present it is one of the major epidemic diseases of Kashmir.

During the winter of 1947-48 there were two small outbreaks. One started in December 1947 in the refugee camps at Baramula and Srinagar and the other in March 1948 at village Barari Pathri in Badgam tehsil. The present work is a study of these outbreaks.

1947-48 OUTBREAKS.

Course of the epidemic.—Most of the refugees reached Srinagar and Baramula in the third week of November 1947. The first known case (Rangil Singh) fell ill within 3 weeks of his arrival in Srinagar. During his illness he was visited by Ram Singh from Baramula, who contracted the disease during his stay and died a fortnight after his return to Baramula. Further cases in both these places were contacts of these two patients.

Total cases in Srinagar were 28, confined to 5 families in 3 neighbouring camps, whose members were related and paid frequent visits to each other. Lack of contact with other families and camps restricted the further spread of infection. Moreover, most of the refugees were from endemic areas and were probably immune due to previous infection. In one family of 4 adults and 5 children, only the children got typhus one after the other. This family had come from village Dardpura near Gulmarg where, in an outbreak in 1945, all the adults suffered from it. At Baramula there were 25 cases in refugee-houses and one in the hospital staff.

De-lousing with DDT and vaccination of all the refugees was started at the end of March, simultaneously at Baramula and Srinagar. This effectively controlled the outbreak and prevented its spread in the local population, also no case occurred in the refugees after the first week of April.

In the village of Barari Pathri there were only 10 cases in 3 houses, all at the end of March and April. One of these cases, however, was murine typhus. The previous outbreak here was in March 1945.

Out of a total of 62 cases, 53 were adults and nine children. In adults 26 were males and 27 females. The fatal cases were 5 males between the age of 30 and 35 years and one female aged 60 years. All the children survived. The mortality in adults was 11.3 per cent.

Clinical.—The symptoms given below are a summary of 53 cases in adults and 9 in children.

Adults.—The onset was generally sudden with rigor, aches in the limbs, headache and rarely vomiting. The cases were mostly seen towards the end of the first week when the face was flushed, eyes were congested and the patient had a stuporous look. The tongue was dry and coated, in serious cases covered with dark sordes, tremulous and was brought out slowly. Insomnia was invariably present. Between the fifth and the ninth days the patient was most toxic, mental dullness was common; muttering delirium started in 6 cases, out of which 4 were fatal. Generalized eruption appeared during this period in 50 per cent of the cases. The rash was discrete, macular, in some cases circumscribed and in others the margins gradually faded out. It was most marked on trunk and limbs, sometimes there were spots on the face and neck also, but palms and soles were always free. The staining disappeared during convalescence or even earlier. The spleen was soft and palpable in 8 cases and out of these tender in one case; liver was palpable

in one case, but mild jaundice developed in two cases. Three cases complained of constipation and gastric pain. Slight bronchitis was present in 5 cases. Deafness was complained by 3 cases, but probably it was present in others also, for generally one had to speak loudly to the patient. The fever lasted for 10 to 22 days and mostly came down by lysis. The fatal cases died towards the end of the second week. The complications observed were parotitis in a father and son; anuria in one case for 36 hours at the end of the first week; and hysteric fits during crisis in a girl aged 20 years.

Children.—In children below the age of 14 years the disease was very mild. The onset was more gradual, headaches and aches in the body were much less or absent. Rash appeared in one case out of 9, the fever lasted for a shorter period, convalescence was brief. Rather typhus was only suspected due to the presence of other cases in the house.

Laboratory diagnosis.—The Weil-Felix reaction was of the same pattern as reported by Felix (1933), i.e. high OX19, low OX2 and negative for OXK. The only exception was one case in which the first specimen taken in the beginning of the second week was positive in higher dilution for OX2 than for OX19, but subsequently OX19 went up much higher. In three cases the OX19 titre remained 1:80 or below. To find out the basic level of the community for OX19 agglutinins, the blood of 23 contacts who had escaped from infection was examined. Two were positive in a titre of 1:40, three 1:20 and the rest were negative.

Complement-fixation tests were carried out with the sera of 23 cases by the Base Typhus Research Laboratory at Poona; seven cases were positive in higher titre with epidemic than with murine, and 15 were positive with epidemic antigen only. One case from Barari Pathri reacted like murine typhus, it was positive in higher titre with murine antigen and in lower with epidemic (Table I):—

TABLE I.

Weil-Felix and complement-fixation reactions of some of the human sera.

Case.	Weil-Felix.		COMPLEMENT-FIXATION TEST.									
			Epidemic.					Murine.				
	OX19	OX2	10	20	40	80	160*	10	20	40	80	160
S. Singh ...	1,280	20	4	4	4	4	4	0
P. Kaur ...	640	320	4	4	4	4	4	0
L. Singh ...	80	80	4	3	2	0	...	0
H. Kaur ...	1,280	80	4	4	4	4	4	4	4	2	2	0
Sadq ...	1,280	40	4	4	4	4	4	4	4	2	2	0
Miriam ...	320	80	4	3	2	0	...	4	4	4	4	3

* Dilutions higher than 1:160 were not put up.

Strains isolated.—The bloods of five cases were inoculated into guinea-pigs. Rickettsial strains were recovered from three, while two attempts were unsuccessful. Two strains were recovered from lice: one batch was collected from a case at Baramula and another from a case at Barari Pathri.

Reactions in guinea-pigs (human strains).—There was no febrile response in 3 out of 43, i.e. 7 per cent of the guinea-pigs; but rickettsia were seen in smears from these non-reactors and their brain passaged into the next animal produced fever. All the three strains were orchitic to begin with but lost this property after 4 to 7 passages. In the first 7 passages the tunica reaction developed in 12 out of 23 animals. This change could be due to two factors: (a) that the passages were carried out with brain tissue and not the scrapings from the tunica, and (b) that due to shortage of animals young guinea-pigs had to be used in later passages.

Louse strains.—The two strains isolated were non-orchitic from the very beginning, but both produced ascites in 3 to 4 early passages but not later on. Silent infection similar to human strains was present in 5 out of 30, i.e. 16 per cent of the animals.

Complement-fixation test.—The reaction in guinea-pigs was variable similar to the cases. Even with the same strain some were positive with epidemic antigen only and others in higher titre with epidemic and in low titre with murine antigen.

Cross-immunity tests.—The test was carried out between a lice and a human strain, using 8 protected and 4 control guinea-pigs with each. The protected animals did not react to a challenge dose of the other strain. The four controls with the human strain reacted, but one out of four with the lice strain failed to show a febrile response. Still one can say that the two strains were immunologically identical.

Incidence and mortality in Kashmir.—The statistics in the Medical Administrative Reports of Kashmir and Jammu State (1943-47) are only factual so far as the typhus group of fevers are concerned because murine and epidemic typhus are grouped under the same heading. This error was unintentional for the presence of murine typhus was never realized. However, from the epidemic year of 1943 onwards the incidence shows a decline (Table III). The seasonal incidence as shown by these figures is high in early winter and spring (Table II):—

TABLE II.

Monthly incidence of typhus from 1945 to 1948.

Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.
43	51	67	214	11	29	9	5	1	0	0	93

The reporting of morbidity in the State has always been more faulty than that of deaths, particularly during the most severe part of the winter. Therefore, the seasonal incidence for mid-winter is not true and the mortality rates are higher

than actual. According to the Administrative Reports the mortality rate varied from 26.4 to 33, while in small outbreaks investigated by individuals the range was from 2.3 to 18.7 (Table III). The later figures are considered as more accurate.

TABLE III.
Number of cases and deaths from different records.

Source.	Year.	Cases.	Deaths.	Mortality rate.
Administration Report ...	1943	1,526	408	26.7
Administration Report ...	1944	966	330	33.1
Administration Report ...	1945	127	43	33.8
Administration Report ...	1946	320	91	28.4
Administration Report ...	1947	20	6	30.0
Kaul (1944)	108	20	18.7
Jackson (1945)	...	213	5	2.3
Hussain (1945)	...	106	20	16.8
Present authors (1949)	...	62	6	9.6

Effect of typhus vaccine.—Nine voluntary workers were engaged in de-lousing the refugees in infected camps. Six of them were protected, while three had refused vaccination. One of the latter got a mild attack of typhus. Jackson (1945) mentions one mild case in vaccinated persons at Gilgit, but the number vaccinated is not given. Hussain (1945) records 16 cases in 252 vaccinated persons in Kashmir, of these 6 had one, 9 had two and 2 had three inoculations each, but none of them died. As a comparison he mentions 106 cases with 20 deaths. Vaccine has been used extensively in the State since 1944 but the above is the only data available, and even this indicates that the vaccine has been of value in lowering the mortality.

CONCLUSION.

The outbreak in refugees was typical of epidemic typhus in all its aspects. The conditions for heavy louse infestation were favourable due to extreme cold, lack of change of clothing, lack of fuel and facilities for a bath. The cases were confined to contacts and the spread was controlled by de-lousing. The symptoms resembled epidemic typhus and the strains isolated from patients and lice reacted similar to *R. prowazeki* infection in guinea-pigs.

SUMMARY.

A limited outbreak of epidemic typhus is described. The course of the epidemic and the symptoms of the disease were typical of epidemic typhus.

The reactions in guinea-pigs, of the strains of rickettsia isolated from cases and lice, were similar to *R. prowazeki* infection.

The authors owe their thanks to the Director of Health Services, and the Epidemiologist, Jammu and Kashmir State, for their help; also to Major C. Speechly of the Base Typhus Research Team who carried out all the complement-fixation tests.

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BIOCHEMICAL INVESTIGATIONS IN KALA-AZAR.

Parts II and III.

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Part II.

ADRENO-CORTICAL FUNCTION IN KALA-AZAR.

THE disease kala-azar was so named because of the dark pigmentation of the skin that is often seen in the well-developed cases of the disease (*kala* = dark, *azar* or *jwar* = fever). The causation of this increase of pigmentation was, however, not fully investigated in the past. It had been suggested that this accentuation of pigmentation was possibly due to the increased activity of the melanoblasts as there was other evidence of hypo-adrenia, but it was also an intensification of the natural pigmentation due to the dryness of the skin (Napier, 1943). Also De (1934) had reported the finding of parasitized macrophages in the supra-renal cortex in 2 out of 26 cases. Thus, though the adrenal cortex was suspected to be affected and thus to cause the darkening of the skin in kala-azar, no information is available as to the functional status of the adrenal cortex. This is obviously due to the fact that the tests of adreno-cortical function have been developed only during the recent years.

It was in 1932-35 that Loeb and his co-workers (Loeb, 1932; Loeb *et al.*, 1933, 1935) discovered the alteration of the electrolyte balance in the blood in adrenal insufficiency. Since then several tests of adrenal function have been

developed that depend upon the changes in the blood and urine caused by a salt-poor diet. Of the tests evolved on this basis, that first described by Cutler, Power and Wilder (1938) has been found to yield consistent results in the hands of different workers and its value recognized in the detection of minor degrees of adreno-cortical disease where significant changes in the blood are often absent. In the present investigation of the adreno-cortical function in kala-azar, the Cutler-test was, therefore, selected and all precautions to deal with any crisis that might occur in cases with severe adrenal insufficiency were adopted. In addition to the Cutler-test, the plasma-chloride content was estimated at the beginning of the test and in a few cases the effect of administration of desoxy-corticosterone-acetate (DOCA) on the chloride excretion studied. In this paper are presented the results of investigation of the adreno-cortical function with these tests and the results discussed in the light of the clinical features of the cases.

The Cutler-test consists in withdrawing salt from the diet for a period of 52 hours under standard conditions of fluid and potassium intake and measuring the chloride or sodium excretion during the last four hours of the test period. The patients with adrenal insufficiency respond by excreting urine of high chloride content despite the salt deprivation. According to the diagnostic standards of Cutler, excretion of over 225 mg. per cent as chlorine or 370.8 mg. per cent as sodium chloride in the last four-hour sample of urine indicates adrenal insufficiency and an excretion of 125 mg. as (Cl) or 206 mg. as NaCl is a sign of normal function of the adrenal cortex; intermediate values indicate doubtful cases that can be diagnosed by further prolongation of the test period and increased administration of potassium.

In these investigations the procedure of Cutler *et al.* (*loc. cit.*) is followed in detail with two modifications: (i) the exact diet as recommended by Cutler could not be given. We selected a diet that was easily available and would suit the Indian patient. The mineral content of the diet was not directly estimated, but it appears from the charts given by McLester (1944) that the sodium chloride and potassium content of the diet adopted was slightly higher than that of Cutler *et al.*; (ii) the dosage of potassium administered was higher. The dosage of potassium citrate was increased because it was felt that this would bring out slighter degrees of dysfunction better. These modifications were not really any serious handicap, because a series of normal cases was studied in order to obtain the standard values for Indians under the conditions of the test. Slightly modified régime of the Cutler-test followed is given below:—

First day: Blood sample collected for the estimation of plasma-chloride with the patient in the fasting state.

Diet: Milk 32 oz., sugar $1\frac{1}{2}$ oz., potato 4 oz., fish 6 oz., bread (salt-free) 8 oz., banana 1, eggs 2, no salt used in cooking or with food.

Fluid intake: No restriction. The patient is encouraged to drink as much water as he can.

Potassium intake: Potassium citrate 2 gr. per pound of body-weight per day, divided into four doses and given in the form of a mixture.

Second day: Diet as on the first day. Total fluid intake 18 c.c. per pound of body-weight. Potassium intake as on the first day.

Third day: Total fluid intake till 11 a.m., 9 c.c. per pound of body-weight. No food or drink after 11 a.m. Potassium salt omitted.

The patient evacuates the bladder at 8 a.m. and 12 noon and the latter sample, i.e. the urine excreted in the last four hours of the salt-poor diet, is examined for chloride content.

The test was performed in a series of 53 cases of kala-azar admitted under the care of one of the writers (P. C. S. G.) into the Carmichael Hospital for Tropical Diseases, Calcutta. In 51 of these cases the diagnosis of kala-azar was confirmed by the demonstration of the parasite as well as by serum tests. In the other two cases the clinical diagnosis of kala-azar was confirmed by strongly positive complement-fixation reaction. The controls were selected from a group of cases which had been admitted for chronic bowel disease, but were progressing favourably at the time of the test. The excretion of chloride as NaCl in the series of kala-azar cases and the controls are shown in the Graph and in Table I:—

GRAPH.

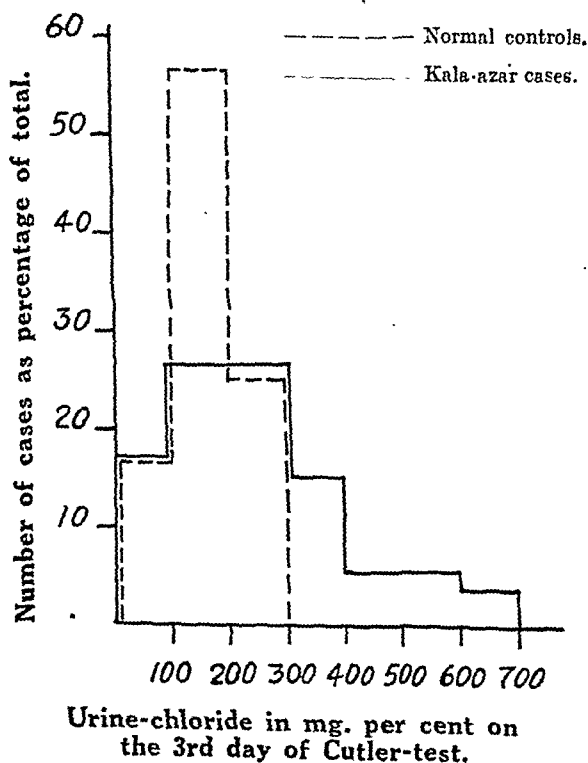


TABLE I.

*Results of Cutler-test in kala-azar cases
and normal controls.*

Urinary chloride as NaCl, mg. per cent.	NUMBER OF CASES.	
	Kala-azar.	Normal.
0 to 100 ...	9	2
101 to 200 ...	14	7
201 to 300 ...	14	3
301 to 400 ...	8	0
401 to 500 ...	3	0
501 to 600 ...	3	0
601 to 700 ...	2	0
TOTALS ...	53	12

The maximum urinary-chloride concentration in the control group was 300 mg. per cent, the mean and standard deviation being 153.0 ± 85.2 mg. per cent. In the kala-azar group the mean and standard deviation was 262.7 ± 158.8 mg. per cent; 16 cases showed chloride excretion well above the highest normal of 300 mg. per cent. Thus, 16 out of 53, i.e. 30.2 per cent of the kala-azar cases, had shown abnormally high chloride excretion under the conditions of Cutler-test. In view of the fact that none of the patients was suffering from diabetes or renal disease, conditions that might interfere with the Cutler-test, the conclusion is justified that this 30 per cent of the cases of kala-azar showed adreno-cortical hypo-function.

On analysing the results obtained in the different stages of kala-azar, it was found that the percentage of positive reactions was 25 for the early cases, 30 for the moderately advanced cases and 33.3 for the well-developed cases (early case—splenic enlargement 2" or less; moderately advanced case—splenic enlargement between 2" and 4"; well-developed case—splenic enlargement more than 4" below the costal margin). There is thus a definite tendency for the test to be more positive as the disease progresses to a more chronic stage. This is also apparent from the average chloride excretion of 437.5 in the early cases and 457.3 mg. per cent in the moderately advanced and the well-developed cases of the group of Cutler-positive cases.

If the results are considered in relation to the standards laid down by Cutler *et al.* (*loc. cit.*) positive reaction is seen in 9 (17 per cent), doubtful reaction in 21 (39.6 per cent), and negative reaction in 23 (43.4 per cent) of the cases. In view,

however, of the slight difference in the present régime of the test, it is more accurate to compare with our normal standards.

PLASMA-CHLORIDE VALUES IN KALA-AZAR.

The plasma-chloride values before salt-poor diet in the kala-azar and the control groups were as follows :—

TABLE II.

Analysis of plasma-chloride values in kala-azar and normal controls.

Plasma-chloride as NaCl, mg. per cent.	NUMBER OF CASES.	
	Kala-azar.	Controls.
471 to 500 ...	1	0
501 to 530 ...	6	0
531 to 560 ...	6	0
561 to 590 ...	22	7
591 to 620 ...	14	3
621 to 650 ...	4	0
TOTALS ...	53	10

Mean \pm standard deviation 583.9 \pm 32.4 mg. per cent and 594.4 \pm 9.4 mg. per cent for the kala-azar and the control groups, respectively.

In the control series, the lowest figure for plasma-chloride was 588 mg. per cent. If this is accepted as the lowest normal value, 43.4 per cent of the kala-azar cases showed subnormal plasma-chloride values varying between 494 mg. and 585 mg. per cent. On comparison with the normal values according to the standards (560 mg. to 630 mg. per cent) laid down by Peters and van Slyke (1931), it was, however, found that in 10 out of the 53 cases, the plasma-chloride values are below normal. Also on consideration of the values in relation to the stage of the disease it was found that in 1 out of 13 early cases, 3 out of 20 moderately advanced cases and 6 out of 20 well-developed cases, the plasma-chloride concentration was below the lowest normal limit of 560 mg. per cent. It is known that the plasma-chloride values may not be appreciably reduced in all cases of adrenal deficiency, e.g. Willson *et al.* (1942) obtained plasma-chloride values between 510.9 mg. and 604.8 mg. per cent in Addison's disease, and that there may be a considerable degree of overlapping between the normal range and that obtained in Addison's disease. On considering the lowering of plasma-chloride values in a proportion of the kala-azar

cases in the light of the positive reactions obtained with the Cutler-test, it is justifiable to consider the lowering of plasma-chloride content as due to adrenal hypo-function.

THE EFFECT OF DESOXY-CORTICOSTERONE-ACETATE (DOCA) ON
ADRENO-CORTICAL FUNCTION IN KALA-AZAR.

Willson *et al.* (*loc. cit.*) reported that patients suffering from Addison's disease who were having DOCA through the procedure of Cutler-test responded like normal persons, whereas other cases that did not have DOCA responded typically by excreting high concentration of chloride in the urine. It was, therefore, thought that the repetition of the Cutler-procedure in some of the positive cases after specific treatment for kala-azar or during administration of DOCA, might throw further light on the adreno-cortical function in kala-azar. Three cases were studied in this manner. The results of the Cutler-test and the plasma-chloride concentration in these cases are given in Table III :—

TABLE III.

	Plasma-chloride as NaCl, mg. per cent, before Cutler-régime.	Urinary chloride concentration as NaCl, mg. per cent, during the last four hours of the Cutler-test.
CASE I:		
Before DOCA	588	500
After DOCA 5 mg. \times 5 days	180
CASE II:		
Before DOCA,	600	650
After insufficient treatment for kala-azar	495	500
After DOCA 10 mg. \times 4 days	300
CASE III:		
Before DOCA	615	585
Immediately after Sb therapy ...	600	1,050
After DOCA 5 mg. \times 6 days	550

It will be evident from Table III that the administration of DOCA caused well-marked decrease of chloride excretion during Cutler-test in 2 cases and a slight decrease in the other. It is possible that the dosage of DOCA was inadequate in case III. This response to the administration of DOCA may be regarded as further evidence of adrenal hypo-function.

CLINICAL FEATURES OF THE CUTLER-POSITIVE CASES.

From an analysis of the clinical features and other biochemical findings of the 16 cases that gave positive results with the Cutler-test, it was found that 12 complained of their complexion becoming darker and the remaining four either complained of pallor or did not notice any change. Pigmentation of the skin and sometimes of the mucous membrane as well was noticed in 14 of the cases. Systolic blood-pressure was low, i.e. below 100 mg. of mercury in 9, and there was hypo-chloræmia, i.e. values below our normal limit of 588 mg. per cent in 6 cases.

It is well known that adreno-cortical hypo-function leads to pigmentation in Addison's disease. In the present series of cases of kala-azar it has been shown that there was biochemically demonstrable adrenal deficiency in a considerable proportion of the cases. It thus appears that adrenal hypo-function is an important factor in the causation of dark-pigmentation of the skin in kala-azar. Low blood-pressure, emaciation, asthenia at times seen in kala-azar cases may also be possibly due to the same cause.

SUMMARY.

1. Adreno-cortical function was studied in a series of 53 cases of kala-azar, Cutler *et al.* (*loc. cit.*) test, the estimation of plasma-chloride and clinical study of the patients being employed for the purpose.

2. On comparison with the results obtained in a series of normal cases, it was found that in 16 out of 53 cases, i.e. 30.2 per cent, of kala-azar in all stages of the disease, positive indication of hypo-function was obtained with the Cutler-test.

3. Plasma-chloride values were found to be subnormal in 43.4 per cent of the cases when compared with the values obtained in normal controls. On comparison with the European standards, only 10 out of 53 cases showed hypo-chloræmia.

4. The administration of desoxy-corticosterone-acetate (DOCA) to three cases led to well-marked decrease of chloride excretion in two and a less marked decrease in the third.

5. On consideration of the above biochemical findings in the light of the clinical features of kala-azar, it is concluded that adreno-cortical hypo-function that is present in a fair proportion of the cases is an important causative factor for dark-pigmentation of the skin and possibly emaciation, low blood-pressure and asthenia seen in kala-azar.

Part III.

OTHER BIOCHEMICAL CHANGES IN KALA-AZAR.

SOME of the biochemical constituents of blood were estimated with a view to find out if any deviation from the normal occurs in kala-azar. The following constituents were studied: urea-nitrogen, non-protein nitrogen (NPN), sugar, chloride, cholesterol and serum-proteins.

Proteins and NPN were estimated by the micro-Kjeldahl method, and urea, sugar, chloride and cholesterol by the methods of Barrett (1935); Folin and Wu (1920), van Slyke (1923) and Bloor *et al.* (1922), respectively.

The diagnosis of kala-azar was confirmed in all cases except three by the demonstration of the parasite by spleen-, sternum- or tibia-puncture in addition to the serum-tests. In these three cases the diagnosis was confirmed by the complement-fixation test done according to the technique described by one of the writers (Sen Gupta, 1945). All the patients were admitted under the care of one of the writers (P. C. S. G.) into the Carmichael Hospital for Tropical Diseases, Calcutta.

The results of estimation of serum-proteins and plasma-chloride have already been discussed in connection with the hepatic and supra-renal functions in Parts I (Chakravarty *et al.*, 1949) and II of this series, respectively. The values relating to urea-nitrogen, non-protein nitrogen, sugar and cholesterol content of blood in the kala-azar patients are presented in Tables IV to VII :—

TABLE IV.

Blood-urea nitrogen values in 44 cases of kala-azar.

Blood-urea N (mg. per cent).			Number of cases.
10.1 to 12	7
12.1 to 14	14
14.1 to 16	16
16.1 to 18	5
18.1 to 20	1
20.1 to 22	1
TOTAL			44

TABLE V.

Blood-NPN in 10 cases of kala-azar.

Blood NPN (mg. per cent).			Number of cases.
21 to 25	2
26 to 30	3
31 to 35	4
36 to 40	1
TOTAL			10

TABLE VI.

Blood-sugar in 30 cases of kala-azar.

Blood-sugar (mg. per cent).	Number of cases.
61 to 70 	2
71 to 80 	3
81 to 90 	10
91 to 100 	11
101 to 110 	2
111 to 120 	2
TOTAL ...	30

TABLE VII.

Blood-cholesterol in 30 cases of kala-azar.

Blood-cholesterol (mg. per cent).	Number of cases.
60 to 100 	13
101 to 140 	11
141 to 180 	5
181 to 220 	1
TOTAL ...	30

The normal values for Indians are as follows (Bose *et al.*, 1946) :—

Blood-sugar—80 mg. to 110 mg. per cent.

„ urea N—8 mg. to 18 mg. per cent.

„ NPN—25 mg. to 35 mg. per cent.

„ cholesterol—100 mg. to 180 mg. per cent.

From Tables IV to VII it will be evident that, apart from the changes in the blood-proteins and chloride content that have already been discussed elsewhere, the other abnormalities noted were as follows : urea-N was above normal in 2 out of 30 cases, NPN was higher than normal in 1 out of 10 cases, sugar was below normal in 2 out of 30 cases and blood-cholesterol below normal in 13 out of 30 cases.

Napier (*loc. cit.*) found hypo-glycæmia frequently in kala-azar, and Greig and Kundu (1925) also found that the blood-sugar of kala-azar cases was usually

low. Hypo-glycaemia found in only 2 out of 30 present cases is at variance with the findings of the above workers. It is possible that they were dealing with more advanced stages of the disease.

Hypo-cholesterolæmia was found in 13 out of 30 cases of kala-azar. On analysing the results of hæmatological and other biochemical investigations carried out in these 30 cases, the following conclusions could be arrived at: (i) there was a significant correlation between the cholesterol content and the hæmoglobin values of blood, the correlation coefficient 'r' being 0.7; (ii) there was no significant difference for cholesterol and hæmoglobin values between the cases showing normal and subnormal function of the adrenal cortex as estimated by the Cutler-test*. Defective hepatic function was seen in both groups showing normal or low cholesterol values and there was no significant difference between the two groups.

SUMMARY.

The results of estimation of urea-N, NPN, sugar and cholesterol content of blood of kala-azar patients are presented. Hypo-cholesterolæmia was found in 13 out of 30 cases. This could be correlated with the hæmoglobin content of blood.

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COMPARATIVE RESPONSE OF MAMMARY GLANDS TO EARLY GONADECTOMY.*

BY

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DURING the experimental studies on breast cancer in mice at these Laboratories it was found necessary to study the rôle of hormones in mammary carcinogenesis. It was, therefore, planned to investigate the comparative response of mammary glands to identical hormone treatment in cancer-susceptible and cancer-resistant strains of mice. To facilitate control of hormonal factor in the experiment, the first essential step was to remove the normal source of gonadal hormones from both the strains soon after birth and to administer an identical dose of extrinsic ovarian hormones at maturity. Some of these animals gonadectomized within thirty-six hours after birth were isolated and raised separately as castrated controls without any hormone treatment. These were studied at the age of seven to eight months and the present paper reports the result of these studies on two strains of mice (C_3H and C_{57} black), after early gonadectomy.

MATERIAL AND METHOD.

The strains used in the experiment were brought over from Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine, U.S.A., in 1941 and the progeny was raised by brother-sister mating for the last eleven generations in the laboratories of Tata Memorial Hospital. Strain C_3H has a high susceptibility to spontaneous cancer of the breast, the reported incidence of spontaneous breast tumours being 95 to 100 per cent in both virgins as well as breeding females; while C_{57} black is considered as a cancer-resistant strain with an incidence of spontaneous breast cancer of less than 0.5 per cent in breeding females. The technique used for gonadectomy of new-borns was simple and essentially the same as the one used by Fekete *et al.* (1941).

* Investigation carried out under the auspices of the Indian Research Fund Association.

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Gonadectomized sucklings were weaned from their mothers after twenty days, and brothers and sisters were segregated at maturity. Males and females were sacrificed at the age of seven to eight months and gross mounts of mammary glands were prepared after staining with Kernechtrot. A comparative study of the gross appearance of uterine horns and adrenals was also undertaken. Twenty C₃H females and sixteen C₅₇ females along with eight castrated males from each strain were available for the present study :—

EXPERIMENTAL FINDINGS.

A remarkable difference was noticed in the general condition of the animals of two strains at the age of seven to eight months. Gonadectomized males and females belonging to non-susceptible strain C₅₇ black began to put on weight and looked more obese than the controls. On the other hand, C₃H females appeared weak, emaciated and stunted in growth. Five out of twenty died before attaining the age of seven months. Animals from both the strains were weighed before they were killed. The average body-weights of gonadectomized males and females in the two strains as against the normal controls of the same age are given in Table I.

TABLE I.

Strain.	Sex.	Normal control. Average weight (g.).	Gonadectomized average weight (g.).
C ₃ H	Male	26.2 ± 0.82	24.0 ± 0.76
	Female	24.4 ± 0.63	21.0 ± 0.12
C ₅₇	Male	24.8 ± 0.74	31.2 ± 0.84
	Female	25.2 ± 0.33	31.8 ± 0.44

The average body-weights of gonadectomized animals thus differed from normal average range. Considerable difference was noticed between weights of two strains after ovari-ectomy although both the strains were raised under identical laboratory conditions.

It was surprising to observe that on a daily vaginal smear examination of ovari-ectomized females between the ages of seven and a half to eight months, a cornification of vaginal epithelial cells was demonstrable in C₃H females. Sixteen

out of twenty C_3H females were found to go into regular estrus at this late age. On the other hand, fourteen out of sixteen C_{57} females did not show keratinization of the vaginal mucosa. The uterine horns of fifteen out of twenty C_3H females showed evidence of estrogenic stimulation and resembled the uteri of normal intact virgins. In C_{57} black, on the other hand, the uterine horns were seen as thin thread-like structures indicating an absence of estrogenic stimulation.

Eleven out of twenty C_3H females developed nodular outgrowths on their adrenals which were absent in C_{57} black females.

Although a development of mammary glands was not expected in these animals which were gonadectomized at birth, the gross mounts showed a peculiar mammary architecture in strain C_3H . A large majority of C_3H females (16/20) developed unusually long slender mammary ducts branching sparsely (Plate III, fig. 1). The ductal pattern resembled that occurring in normal females of the same age. The acinar proliferation, however, was completely absent. The sparse branching of mammary ducts and further degree of dense ductal branching in strain C_3H resembled ductal patterns α and β described in a previous publication (Khanolkar and Ranadive, 1947). Acinar structures were totally absent in nineteen out of twenty C_3H females, condition represented by acinar type 'A'. Only one C_3H female developed focal acinar structure of type 'C' described previously (*loc. cit.*).

The C_{57} black females did not show any mature breast development after early ovari-ectomy. Five out of sixteen such animals showed a couple of rudimentary mammary ducts without side offshoots or acinar structures (Plate III, fig. 2). The rest of them did not give evidence of breast development. A remarkable difference was thus noticed in the breast development of the two strains after early ovari-ectomy.

All the sixteen males studied from strains C_3H and C_{57} black did not show any sign of breast development with the exception of one C_3H male in whom a stunted mammary gland with few hyperplastic nodules was seen.

In addition to ductal proliferation in strain C_3H described above, heavy inorganic deposits were observed in the stroma of the glands (Plate III, fig. 3). These deposits were more or less uniformly scattered between branches of mammary ducts, and were noticed in fifteen out of twenty C_3H females and three out of eight C_3H males, but in only one out of sixteen C_{57} females and in none of the C_{57} males. The material was first suspected to be crystalline cholesterol formed as a result of fat necrosis in C_3H females. A qualitative test for cholesterol according to Bloor's modified method gave negative results.

The whole mounts were then stained with hæmatoxylin instead of Kernechtrot. The deposits turned blackish blue suggesting the presence of calcium salts. A qualitative test for calcium by Clark's method proved the presence of calcium. A simple but fairly sensitive gypsum reaction was also performed. It is a micro-chemical test for identification of calcium in the deposits. On a clean slide a microdrop of 1/10 normal hydrochloric acid was first added to a small quantity of the crystalline deposit and later another microdrop of 1/10 normal sulphuric acid was added to it. Typical needle-shaped crystals of calcium sulphate appeared

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on the slide as a result of chemical reaction and presence of calcium in the deposits was thus confirmed (Plate III, fig. 4).

The findings stated above are described in Tables II, III and IV :—

TABLE II.

Experimental data on C₃H females ovari-ectomized at birth.

Animal number.	Age (days).	Weight (g.).	Mammary pattern.	Uterine horns (appearance).	Adrenals (appearance).
1*	150	21	D	Castrate type	Normal.
2*	137	20	D	"	Nodules present.
3*	195	20	D	"	Normal.
4	240	21	α , A, D	Intact virgin	"
5	240	20	β , A, D	"	"
6	240	22	β , A, D	"	Nodules present.
7	240	21	α , A, D	"	Normal.
8*	135	18	D	Castrate type	"
9*	138	23	α , A, D	"	"
10	238	20	α , A	Intact virgin	Nodules present.
11	237	21	α , A	"	Normal.
12	240	21	α , A, D	"	"
13	238	22	α , A, D	"	Nodules present.
14	238	20	α , C, D	"	"
15	240	22	β , A	"	"
16	235	25	β , A	"	"
17	240	22	α , A	"	"
18	238	20	β , A, D	"	"
19	240	22	β , A, D	"	"
20	240	20	α , A, D	"	"

* Five C₃H females that had to be killed before seven months did not show evidence of estrogenic stimulation. Heavy deposition of calcium salts was present in these females.

R = Rudimentary ducts,

• Ductal patterns designated as α and β , and

Acinar types designated as A and C are described and illustrated in previous publication (Khanolkar and Ranadive, *loc. cit.*).

D = Deposits of calcium salts present in the breast parenchyma.

PLATE III.

ENTIRE MAMMARY GLANDS CASTRATED CONTROLS. (FEMALES)

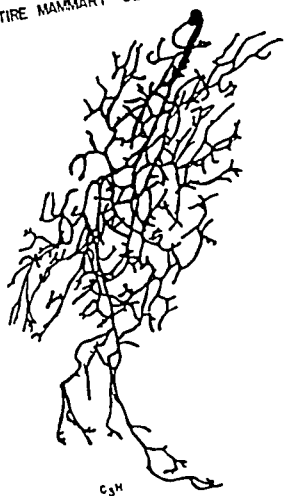


FIG. 1—Silhouette of the first thoracic mammary gland from the right side of an ovariectomized $C_{57}H$ female, 210 days to 240 days old. Enlarged $\times 4$ times.



FIG. 2—Silhouette of the first thoracic mammary gland from the right side of an ovariectomized $C_{57}H$ female, 210 days to 240 days old. Enlarged $\times 4$ times.

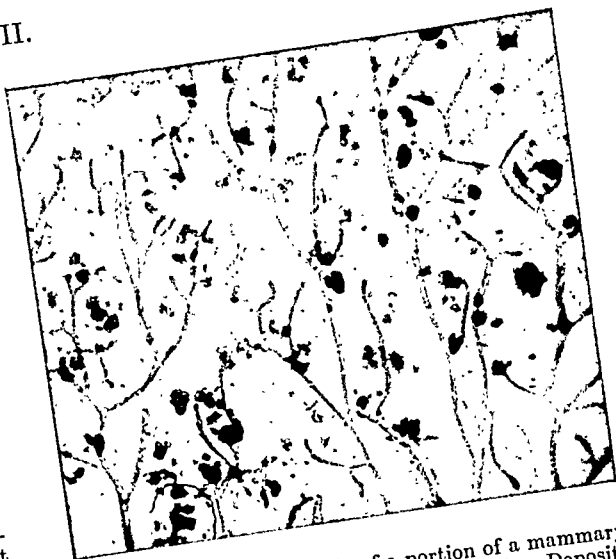


FIG. 3—Photomicrograph of a portion of a mammary gland from a $C_{57}H$ female castrated at birth. Deposits of calcium salts in the breast parenchyma as seen under a low-power microscope, $\times 19$.

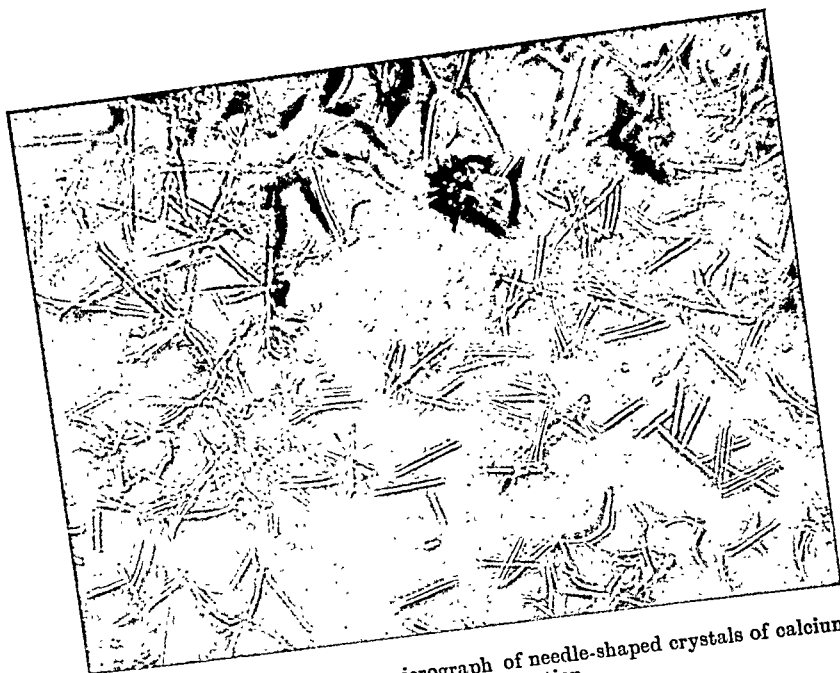


FIG. 4—A low-power photomicrograph of needle-shaped crystals of calcium sulphate formed as a result of gypsum reaction.

TABLE III.

Experimental data on C₆₇ females ovari-ectomized at birth.

Animal number.	Age (days).	Weight (g.).	Mammary pattern.	Uterine horns (appearance).	Adrenals (appearance).
1	237	28	R, A, D	Castrate type	Normal.
2	240	32	R, A	"	"
3	230	30	...	"	"
4	235	32	...	"	"
5	235	35	...	"	"
6	238	28	α , A	Intact virgin type	"
7	240	35	R, A	Castrate type	"
8	240	32	R, A	"	"
9	240	35	...	"	"
10	235	32	...	"	"
11	238	30	...	"	"
12	240	34	...	"	"
13	240	32	...	"	"
14	235	28	...	"	"
15	235	30	...	"	"
16	235	35	...	"	"

R = Rudimentary ducts,

Ductal patterns designated as α and β , andAcinar types designated as A and C are described and illustrated in previous publication (*loc. cit.*).

D = Deposits of calcium salts present in the breast parenchyma.

TABLE IV.

Sex.	Strain.	Total number of animals studied.	Average weight (g.).	Number of animals showing breast development.	Number of animals showing calcium deposits.	UTERINE HORNS.		ADRENALS.	
						Castrate type.	Intact virgin type.	Normal.	Nodular.
Female ...	C ₃ H	20	21.0±0.12	16	15	5	15	9	11
	C ₅₇	16	31.8±0.44	5 (R)	1	15	1	16	...
Male ...	C ₃ H	8	24.0±0.76	1	3	6	2
	C ₅₇	8	31.2±0.84	8	...

DISCUSSION.

Animals gonadectomized at birth presented interesting changes at the age of seven to eight months. The ovari-ectomized C₃H females indicated estrogenic stimulation by a development of uterus and keratinization of vaginal mucosa as well as by growth and development of mammary glands. There was no evidence of such stimulation in C₅₇ black females.

Perry (1938) first noticed positive vaginal smears in old mice spayed at birth. Loeb and Kirtz (1939) later studied response of accessory genital organs to pituitary transplantation after early ovari-ectomy in strain 'D' mice. Woolley, Fekete and Little (1940) observed a marked hypertrophy of adrenal cortex following the development of mammary tumours in strain dba castrates. A comparative study of the effect of early gonadectomy on accessory sex organs of susceptible and non-susceptible strains of mice was also undertaken in the same laboratory. Mammary tumours were observed between the ages of 14 and 24 months in dba mice spayed at birth. More extensive work, however, has been done on extreme dilution strain 'Ce', because of the peculiar anatomical position of adrenals and constitutional hypogenitalism in the strain (Fekete and Little, 1945; Woolley and Little, 1945). Carcinomas of adrenal cortex occurred very frequently in gonadectomized males and females of this strain at an early age of six to seven months. A growth and development of accessory sex organs occurred following the appearance of adrenal cortical tumours in gonadectomized animals. Such observations suggested some relationship between adrenal tumours and development of accessory sex organs.

Our observations on ovari-ectomized C₃H females gave evidence of growth and development of breast consisting exclusively of ductal proliferations. The development of breast in strain C₃H was generally accompanied by nodular overgrowth

of adrenal cortex. Both the breast development and cortical hyperplasia were absent in strain C₅₇ black. Such observations indicated an essential relationship between hyperactivity of adrenal and the development of vagina, uterus and mammary glands in the event of early gonadectomy. Although the animals in the present experiment were studied at a comparatively early age (7 to 8 months) there was a definite indication of estrogenic stimulation in the susceptible strain. It, therefore, appears that, as indicated in the literature (Woolley *et al.*, 1939 to 1945), the breast development in ovari-ectomized C₃H females may have been induced by hormones of adrenal cortex. Nodular overgrowth of the cortex suggested hyperactivity of adrenals. Hyperplastic tissue may produce extra-ovarian estrogenic compounds, and stimulate uterine development and profuse ductal proliferation exclusive of alveolar structures. Adrenals in the non-susceptible strain C₅₇ black do not appear to compensate for the function of absent gonads. Such observations invite further detailed study of gonad-adrenal inter-relationship in the event of early gonadectomy.

Accumulation of calcium deposits in the breast parenchyma of ovari-ectomized C₃H females has not been reported in the available literature. The only plausible explanation that can be put forth for these deposits is that hormone deficiency in susceptible castrated controls may affect calcium metabolism of the animals leading to a heavy deposition of calcium salts. It appears that hormone deficiency does not affect calcium metabolism in the non-susceptible strain C₅₇ black as the strain is normally used to scanty hormonal stimulation. It will be worth while investigating the chemical composition of bones of these castrated controls. The result of the investigation may throw some light on changes in the calcium metabolism following gonadectomy.

SUMMARY.

1. Males and females of strains C₃H and C₅₇ black gonadectomized at birth were isolated for study. Response of mammary glands, uterus, vagina and adrenals to gonadectomy was investigated at the age of seven to eight months.
2. Accessory sex organs in C₃H females gave evidence of estrogenic stimulation; C₅₇ females on the other hand presented characteristic castrate type of secondary sex organs. Hyperplasia of adrenal cortex may compensate for function of missing ovaries in the susceptible strain.
3. Heavy calcium deposits were observed in the breast parenchyma of strain C₃H females. Such deposits were rare in strain C₅₇ black.
4. Preliminary findings invite further study of (i) gonad-adrenal inter-relationship and (ii) chemical composition of bones in castrated controls.

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EFFECT OF FOSTER-NURSING ON MATURATION OF OVARIES.*

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SINCE the discovery of extra-chromosomal milk-borne tumour agent in 1936 (Bittner) abundant evidence has been collected revealing a remarkable influence of mother's milk on mammary carcinogenesis in mice (Bittner, 1936 to 1941; Andervont and McEleny, 1939; Murray and Little, 1939). Investigations have also been directed towards a study of the physical and chemical nature of the milk substance and its distribution in the viscera (Bittner, 1941; Bryan *et al.*, 1942). The mode of action of the milk-borne substance is not yet determined.

During the experimental studies on breast cancer at these laboratories a peculiar disturbance in the hormonal metabolism was observed in some female mice after foster-nursing. They showed a consistent alteration in the length of sex cycle and the duration of estrus. It was, therefore, proposed to study the effect of foster-nursing on the hormonal metabolism of different strains of mice.

The problem could have been easily attacked through a study of estrus cycles after cross-suckling. Daily examination of vaginal smears had already been undertaken on a small group of experimental females but this method of approach to the problem was later given up for two reasons. The literature on the subject of sex cycles in strains of mice appeared very confusing. Several investigators (Lacassagne, 1934; Bonser, 1935; Moskop *et al.*, 1935; Burns *et al.*, 1936; Harde, 1934; Deringer *et al.*, 1945, and others) had studied estrus cycles in cancer-susceptible and cancer-resistant strains of mice, and most of them were unable to establish a definite correlation between the duration of estrus and the incidence of spontaneous mammary tumours. It was also thought that stimulation of vaginal mucosa by daily introduction of cotton-swabs for obtaining the material for the smears may affect the rhythmic changes during the sex cycle. The vaginal smear examination was, therefore, replaced by a method of hormone estimation through the study of maturation of ovaries.

Taylor and Waltman (1940) and Fekete (1946) studied serial sections of ovaries of cancer-susceptible and cancer-resistant strains of mice at different age periods.

* Investigation carried out under the auspices of the Indian Research Fund Association,

They observed differences in the average number of corpora lutea in the two strains studied by them, and interpreted such variations as due to differences in hormonal stimulation. It was, therefore, planned to study the effect of foster-nursing on the number of corpora lutea in the ovaries.

MATERIAL AND METHOD.

Ovaries were selected from some foster-nursed female mice which had been studied previously for mammary architecture at the age of seven to eight months (Khanolkar and Ranadive, 1947). These animals belonged to three different strains: cancer-susceptible strains, C₃H and strong 'A' and cancer-resistant, C₅₇ black.

Complete estrus cycles were traced twice a month from maturity (2½ months) to the age of eight months in the first set of thirty-five females. A simple cotton-swab method was used for vaginal smears and smear spreads were stained with polychrome methylene blue and classified according to their cell contents.

One hundred and seventy-one ovaries from 94 females isolated in nine different groups were fixed in Bouin's fluid and serial sections 5 μ in thickness were cut and stained with hæmatoxylin and eosin. Special care was taken to see that all ovaries were embedded and cut in almost the same plane. Ten to twenty sections from each ovary were examined, the largest available section was selected for camera-lucida projection and drawn at the same magnification. Outlines of corpora lutea were traced and their number was noted. As weights and volumes were not determined in the fresh material a sum of the two largest perpendicular diameters gave only a rough estimate of the size of the ovary magnified $\times 80$ times.

EXPERIMENTAL FINDINGS.

Normal sex cycle in mice lasts for four to six days, of which the period of heat (estrus) and the period of rest (diestrus) continues for one and a half to two days each (Allen *et al.*, 1935). The length of the sex cycle and the duration of estrus and diestrus were studied in thirty-five female mice. The results are given in Table I:—

TABLE I.

Strain.	Nursed by strain.	Total number of virgins studied.	NUMBER OF ANIMALS SHOWING :		
			Normal cycle.	Long estrus.	Long diestrus.
C ₃ H ...	C ₃ H	6	2	4	...
C ₅₇ black ...	C ₅₇ black	6	6
C ₃ H ...	C ₅₇	10	7	1	2
C ₅₇ ...	C ₃ H	13	3	10	...

Although the number of animals was too small to draw definite conclusions, it was observed that majority of non-susceptible C_{57} females nursed on their own mothers or C_3H females fostered on C_{57} mothers had a normal sex cycle. On the other hand, most of C_3H controls as well as C_{57} virgins foster-nursed on C_3H mothers showed a 'long estrus' phase (see Tables II to XI):—

TABLE II.

STRAIN.	Animal number.	SIZE OF THE OVARY ENLARGED $\times 80$ (SQ. CM.).		NUMBER OF CORPORA LUTEA.		Mammary pattern.
		Large.	Small.	Large.	Small.	
C_{57}/C_{57} (12) ...	1	126.0	95.2	2	2	α , A
	2	174.3	110.9	5	4	α , A
	3	214.5	150.0	5	4	α , A
	4	163.9	157.3	4	2	α , A
	5	77.0	...	4	...	α , A
	6	182.0	112.2	6	5	α , A
	7	191.4	177.1	7	6	β , B
	8	95.7	...	3	...	α , A
	9	128.1	121.9	6	4	α , A
	10	115.2	98.0	5	4	γ , B
	11	143.5	135.1	3	3	α , A
	12	153.3	130.2	8	6	β , B
TOTALS ...		1,764.9	1,281.9	58	40	
AVERAGE ...		147.0	128.7	4.8	4.0	
		137.9		4.4		
		(Average size)		(Average number)		

Effect of Prolonged Nursing on Maturation of Ovaries.

TABLE III.

Species.	Animal number.	SIZE OF THE OVARY ENLARGED $\times 80$ (SQ. CM.).		NUMBER OF CORPORA LUTEA.		Mammary pattern.
		Large.	Small.	Large.	Small.	
C ₅₇ /C ₃ H (13)	13	85.0	70.98	8	7	γ , C'
	14	192.07	138.7	10	10	α , A
	15	176.08	112.8	11	10	α , C'
	16	170.0	107.5	12	10	γ , C
	17	144.3	76.26	12	7	γ , A
	18	198.4	...	10	9	γ , C
	19	159.2	159.5	9	8	γ , C'
	20	202.5	190.32	10	9	α , B
	21	100.0	...	10	...	α , C'
	22	153.27	143.10	13	11	α , A
	23	196.55	188.60	13	13	α , B
	24	145.20	106.14	9	8	β , C'
	25	166.95	...	5	...	α , A
TOTALS	...	2,089.21	1,293.7	132	102	
AVERAGE	...	160.1	129.3	10.1	9.2	
		144.7 (Average size)		9.6 (Average number)		

TABLE IV.

STRAINS.	Animal number.	SIZE OF THE OVARY ENLARGED \times 80 (SQ. CM.).		NUMBER OF CORPORA LUTEA.		Mammary pattern.
		Large.	Small.	Large.	Small.	
C ₂₇ /A (14) ... {	26	94.50	54.40	2	2	α , A
	27	123.50	87.87	2	1	β' , B
	28	87.32	...	5	4	α , B
	29	171.52	95.70	8	7	α , A
	30	221.00	175.50	7	7	α , A
	31	119.00	106.65	6	6	α , A
	32	103.32	99.16	3	5	α , A
	33	162.50	156.00	7	5	β , C
	34	111.10	87.00	5	5	α , B
	35	95.00	76.23	7	5	γ , C'
	36	173.46	148.50	6	4	α , C'
	37	194.50	182.50	6	5	α , B
	38	198.40	165.10	8	7	α , A
	39	150.00	...	8	...	α , A
TOTALS ...		2,008.0	1,434.61	80	63	
AVERAGE ...		143.4	119.5	5.7	4.8	
		131.4 (Average size)		5.2 (Average number)		

TABLE V.

STRAIN.	Animal number.	SIZE OF THE OVARY ENLARGED $\times 80$ (SQ. CM.).		NUMBER OF CORPORA LUTEA.		Mammary pattern.
		Large.	Small.	Large.	Small.	
C ₂ H/C ₃ H (10)	40	132.00	123.12	12	9	α , C
	41	109.14	101.70	9	8	α , A
	42	85.50	...	5	...	α , A
	43	103.50	...	5	...	α , C
	44	170.00	128.48	10	10	β' , C'
	45	168.19	142.00	19	13	β' , A
	46	132.84	...	15	...	α , C
	47	83.60	...	8	5	α , A
	48	92.20	83.46	12	6	α , C
	49	154.00	100.80	15	13	α , C
TOTALS	...	1,230.97	679.56	110	64	
AVERAGE	...	123.9	113.26	11.0	9.1	
		118.6 (Average size)		10.1 (Average number)		

TABLE VI.

STRAIN.	Animal. number.	SIZE OF THE OVARY ENLARGED $\times 80$ (SQ. CM.).		NUMBER OF CORPORA LUTEA.		Mammary pattern.
		Large.	Small.	Large.	Small.	
C ₄ H/C ₃₇ (12)	50	88.32	50.73	4	3	α , A
	51	35.40	51.60	5	3	α , A
	52	77.00	55.20	10	3	β , B
	53	180.00	86.40	6	5	α , B
	54	95.40	94.71	7	5	α , B
	55	121.80	82.95	6	2	α , C
	56	138.00	116.15	5	3	α , B
	57	193.80	180.00	7	5	α , B
	58	88.20	44.00	7	4	α , B
	59	110.96	100.48	5	3	α , A
	60	149.50	137.50	3	2	α , A
	61	158.40	...	4	...	α , A
TOTALS	...	1,436.78	1,002.72	69	38	
AVERAGE	...	119.73	91.15	5.7	3.4	
		105.4 (Average size)		4.5 (Average number)		

TABLE VII.

STRAIN.	Animal number.	SIZE OF THE OVARY ENLARGED $\times 80$ (SQ. CM.).		NUMBER OF CORPORA LUTEA.		Mammary pattern.
		Large.	Small.	Large.	Small.	
C ₃ H/A (12) ...	62	94.86	...	7	5	α , A
	63	90.10	83.95	4	3	α , A
	64	91.91	83.30	3	2	α , A
	65	173.41	165.60	9	6	α , B
	66	83.82	52.50	3	2	α , B
	67	126.0	67.24	4	3	α , A
	68	168.0	88.00	4	3	α , A
	69	120.32	157.50	8	5	α , A
	70	288.80	125.40	5	5	α , C
	71	118.72	146.90	7	5	α , A
	72	85.80	105.30	1	0	β' , A
	73	67.08	59.84	2	1	α , A
TOTALS ...		1,508.82	1,135.53	57	40	
AVERAGE ...		125.73	103.23	4.7	3.3	
		114.50 (Average size)		4.0 (Average number)		

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TABLE VIII.

STRAIN.	Animal number.	SIZE OF THE OVARY ENLARGED $\times 80$ (SQ. CM.).		NUMBER OF CORPORA LUTEA.		Mammary pattern.
		Large.	Small.	Large.	Small.	
Milk.						
A/A (9)	74	89.50	...	5	...	α , A
	75	97.20	60.0	8	4	β , B
	76	126.00	109.25	6	6	γ , B
	77	121.50	88.50	7	3	α , A
	78	191.52	182.20	8	6	γ , B
	79	101.92	101.85	5	2	α , A
	80	140.25	133.00	9	8	α , B
	81	78.30	70.40	5	3	α , A
	82	80.75	...	5	...	β' , B
TOTALS	...	1,026.94	745.20	58	32	-
AVERAGE	...	114.10	102.17	6.4	4.4	-
		108.13 (Average size)		5.4 (Average number)		

TABLE IX.

STRAIN.	Animal number.	SIZE OF THE OVARY ENLARGED $\times 80$ (SQ. CM.).		NUMBER OF CORPORA LUTEA.		Mammary pattern.
		Large.	Small.	Large.	Small.	
A/C ₆₇ (7) ...	83	118.56	84.60	3	2	α , A
	84	241.20	128.80	4	4	α , A
	85	99.06	...	0	...	α , A
	86	61.38	59.85	2	1	β' , A
	87	101.85	74.40	6	5	α , A
	88	158.40	154.40	4	3	γ , B
	89	115.00	72.27	5	5	α , A
TOTALS ...		895.45	574.32	24	20	
AVERAGE ...		127.92	95.72	3.4	3.3	
		111.82 (Average size)		3.35 (Average number)		

TABLE X.

STRAIN.	Animal number.	SIZE OF THE OVARY ENLARGED $\times 80$ (SQ. CM.).		NUMBER OF CORPORA LUTEA.		Mammary pattern.
		Large.	Small.	Large.	Small.	
A/C ₃ H (5) ...	90	173.84	167.20	12	8	α , C'
	91	157.50	...	8	...	β' , C'
	92	157.32	103.68	10	8	β' , C
	93	94.38	91.26	6	4	α , A
	94	128.25	...	10	...	α , C
TOTALS ...		711.29	362.14	46	20	
AVERAGE ...		142.25	120.71	9.24	6.66	
		131.48 (Average size)		7.95 (Average number)		

TABLE XI.

STRAIN. Milk.	Total number of females studied.	Average size of the ovary enlarged $\times 80$ (sq. cm.).	Number of corpora lutea per largest section available (average).
GROUP I:—			
C_{57}/C_{57} ...	12 (22)	137.9 ± 10.3	4.4 ± 0.48
C_{57}/C_3H ...	13 (23)	144.7 ± 12.0	9.6 ± 0.66
C_{57}/A ...	14 (26)	131.4 ± 22.3	5.2 ± 0.54
GROUP II:—			
C_3H/C_3H ...	10 (16)	118.6 ± 19.5	10.0 ± 1.24
C_3H/C_{57} ...	12 (23)	105.4 ± 12.9	4.5 ± 0.47
C_3H/A ...	12 (23)	114.5 ± 14.7	4.0 ± 0.64
GROUP III:—			
A/A ...	9 (16)	108.1 ± 25.2	5.4 ± 0.77
A/ C_{57} ...	7 (13)	111.8 ± 25.1	3.3 ± 0.72
A/ C_3H ...	5 (8)	131.4 ± 27.2	7.9 ± 1.30

* Figures in the brackets represent exact number of ovaries studied from each group.

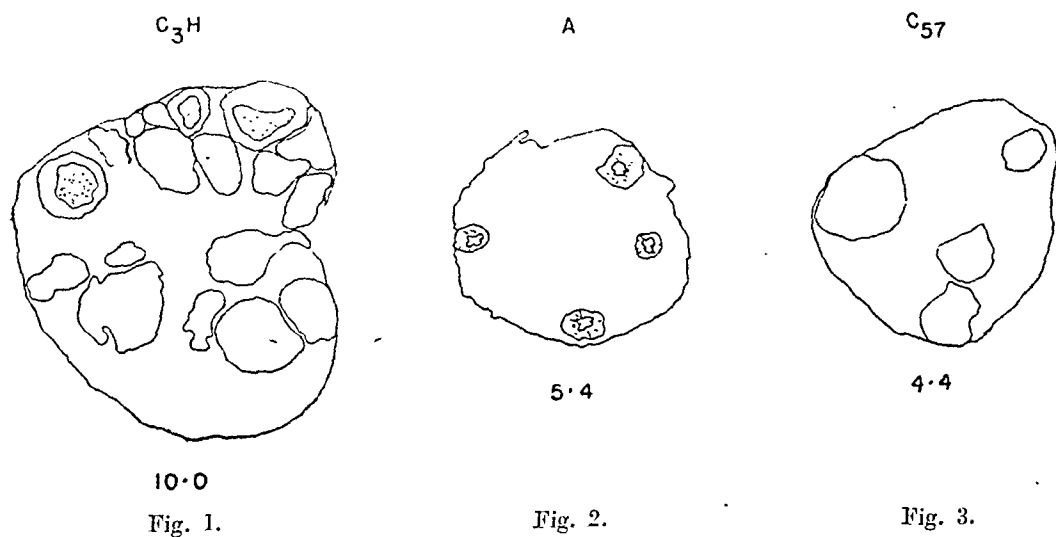
Tables II to X are self-explanatory. They present data about individual observations on 171 ovaries belonging to nine different groups. In a general way the number of corpora lutea seems to run parallel to the complexity of the mammary patterns. The exceptions are underlined.

Table XI summarizes the findings and compares the changes in maturation of ovaries after foster-nursing. The figures for standard deviation indicate great individual variation in the size of the ovaries belonging to the same group. The average size of the non-susceptible ovary appears to be larger than that of the susceptible strains C_3H and A, although the number of corpora lutea in the non-susceptible strain is smaller. The tabulated average size of the ovary is magnified $\times 80$ times the actual size. Besides, the method used for measurement was not very accurate giving only rough estimate of the size. Much significance is, therefore, not attached to these findings. It is decided to weigh fresh ovaries and measure their volumes in future studies. No appreciable change was noticed in the average size of the ovary after foster-nursing.

A remarkable dissimilarity was observed on a general examination of the ovaries belonging to susceptible and non-susceptible strains. The ovaries belonging

to strain C_3H (Fig. 1) were often filled with coalescing corpora lutea so as to completely obscure the stroma, while in strong 'A' (Fig. 2) and C_{57} black (Fig. 3) only few fairly large discrete corpora lutea were seen lying embedded in a relatively abundant stroma. Foster-nursing altered the number of corpora lutea in these strains. In C_{57} black the average number of corpora lutea 4.4 increased to 9.6 when nursed on C_3H and to 5.2 when fostered by strong 'A'. On the other hand, the characteristic number of corpora lutea in strain C_3H decreased from 10 to 4.5 when foster-nursed on non-susceptible C_{57} black and to only 4.0 when nursed on strong 'A'. Very few animals were available for study in the third group. The average number 5.4 of corpora lutea in strong 'A' calculated from only nine females decreased to 3.3 when fostered on C_{57} black and increased to 7.9 when nursed by the susceptible C_3H . Each group of animals thus indicates an alteration in the maturation of the ovary after foster-nursing. The altered structure simulates the characteristic structure of the strain of the foster-mother. Similar observations have already been made with reference to changes in the breast morphology after foster-nursing (Khanolkar and Ranadive, *loc. cit.*). Besides corpora lutea other characters, such as the number and size of follicles, the amount of follicular atresia, etc., offered no immediate points of contrast in different groups.

TEXT-FIGURE.

Effect of foster-nursing on maturation of ovaries.

Camera-lucida projections of sections of ovaries of three strains of mice at the age of 7 to 8 months:

FIG. 1.—Largest available section of ovary of C_3H virgin showing outlines of corpora lutea inside.

FIG. 2.—Largest available section of ovary of strain 'A' virgin showing outlines of corpora lutea inside.

FIG. 3.—Largest available section of ovary of C_{57} black virgin showing outlines of corpora lutea inside.

Figures below drawings give average numbers of corpora lutea in the respective strains.

DISCUSSION.

Since Shinkin and Andervont (1941) proved that foster-nursing does not affect vaginal reaction to extrinsic hormonal stimulation, no other investigator except Armstrong (1948) has attempted to study the direct effect of milk-borne tumour agent on the hormonal metabolism of the suckling.

Armstrong (*loc. cit.*) has recently studied the estrus cycles in a small group of foster-nursed females and has failed to find a statistically significant alteration. Similarly, in the small group of animals studied by vaginal smears in the present series no significant change could be observed after foster-nursing. However, the number of corpora lutea in the ovaries undergoes a significant change after foster-nursing indicating an alteration in the hormonal stimulation.

It would be interesting to determine in larger series whether the changes in the structure of the ovary which are dependent on the hormonal stimulation are also reflected in an alteration of the mammary pattern. The preliminary findings reported here open up an interesting study on the indirect action of the milk substance on the structure of the mammary glands; and the effect on hormonal metabolism and alteration in ovarian structure during maturation.

SUMMARY AND CONCLUSION.

In an attempt to study the effects of foster-nursing on hormonal metabolism the changes in three strains of mice, C₃H, strong 'A' and C₅₇ black, were observed. Serial sections of 171 ovaries from 94 virgins belonging to 9 different groups were studied. A significant alteration in the number of corpora lutea after foster-nursing indicating a change in hormonal metabolism of the animal was observed. It is suggested that a change in the mammary pattern is correlated with a change in the structure of the ovary.

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STUDIES ON INDIAN EDIBLE OILS.

SESAME OIL.

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SESAME OIL is obtained from the seeds of the sesame plant belonging to the family of *Bignoniaceæ*. Two species of sesame have been distinguished *Sesamum indicum* producing yellow or white seeds and *Sesamum orientale* producing dark seeds. The white variety giving an oil pale in colour is known in commerce as 'suffet til', while the black variety which yields a greater proportion of oil is known as 'tillie'.

Sesame oil is used extensively for edible purposes in the raw condition, especially in South India. It commands a premium over ground-nut oil because of its agreeable odour. The problem of storage and marketing of sesame oil has received very little attention, though it is easily susceptible to spoilage. The admixture (5 per cent) of the sesame oil with hydrogenated fat has been made obligatory by the Government and this has led to the increased use of sesame oil in vanaspati industries. Because of the increased use of sesame oil, it is necessary to make a thorough investigation of the factors affecting its quality, stability and nutritive value. Particular attention has been paid to the factors affecting the quality of the raw oil, as this is the starting material in industries, besides its use for edible purposes. The work is divided into two parts: one, dealing with the property of the oil obtained from different sources and by different methods, their storage and preservation with anti-oxidants, and the other dealing with their digestibility and the stability of vitamin A and carotene when dissolved in them.

Part I.

Samples of sesame oil obtained from factories all over India and also those prepared in the laboratory have been analysed for some of their physical and chemical properties and the results are shown in Table I. In all cases the acidity has been expressed as oleic acid :—

TABLE I.

Some constants of raw sesame oil.

Sample number.	Oil.	Acidity.	COLOUR IN LOVIBOND UNITS.		Peroxide value.	Unsaponifiable.
			Yellow.	Red.		
1	Factory	0.7	5.0	0.6	2.9	0.7
2	„	1.2	5.0	0.8	2.6	1.0
3	„	1.7	5.5	0.5	2.0	1.0
4	„	1.9	5.0	0.7	1.1	1.0
5	„	2.1	6.0	1.3	6.8	0.9
6	„	2.5	6.7	1.0	6.3	0.9
7	„	2.8	7.0	2.0	11.9	0.9
8	„	3.8	7.8	2.0	3.6	1.1
9	„	4.0	6.4	2.0	4.0	0.9
10	„	4.5	7.0	1.5	11.7	0.9
11	„	6.1	8.0	1.5	21.3	0.9
12	„	7.0	9.9	4.0	4.0	1.0
13	Cold-drawn, laboratory oil, white seeds.	0.8	5.0	0.8	0.4	1.0
14	Cold-drawn, laboratory oil, black seeds.	3.1	6.0	1.0	1.3	1.1
15	Hot-pressed, No. 13 ...	1.2	5.1	0.7	0.8	1.0
16	Hot-pressed, No. 14 ...	4.9	8.0	2.0	2.3	1.1
17	Seeds No. 13, stored in gunny bags for three months, cold-drawn.	3.4	7.0	3.0	2.8	1.0
18	Do. stored in nitrogen ...	0.9	6.0	1.0	1.2	1.0
19	Do. stored in carbon dioxide ...	1.4	6.0	2.0	1.6	0.9

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An examination of acidity and peroxide value of samples 1 to 12 shows no definite relationship between the two. Generally speaking, however, samples with high acidity also possess high peroxide value. In the case of samples 13 to 19 the changes in acidity are not accompanied by corresponding changes in the peroxide value. It may be observed, therefore, that the peroxide value increases only after the oil has been extracted and stored and this increase in the peroxide value is greater, the greater the initial acidity of the oil. Thus, free fatty acids (f.f.a.) catalyses the development of peroxides in sesame oil. Greater correlation exists between colour and acidity in the case of sesame oil than in the case of groundnut oil (Ramamurti and Banerjee, 1948). Increase in acidity is accompanied by an increase in the depth of colour. All the samples have almost the same value for unsaponifiable matter which is altered very little by the nature and source of the seed, and the method of extraction of oil. Action of heat on the seeds (both white and black) considerably affects the quality of the oil and the storage of the seeds in an atmosphere of nitrogen or carbon dioxide greatly retards the development of free fatty acids. Nitrogen is more effective than carbon dioxide.

Since sesame oil is sometimes refined, experiments were undertaken to study the factors affecting the quality of the oil on neutralization and refining. The results are given in Table II :—

TABLE II.
The effect of neutralization and refining on the storage property of sesame oil.

Number.	Acidity.	Peroxide value.	INDUCTION PERIOD AT 65°C. IN HOURS :		
			Raw oil.	Neutral oil.	Refined oil.
13	0.8	0.4	9.0	9	8.0
5	2.1	6.8	5.5	7	3.25
7	3.0	11.9	4.75	6	3.3
10	5.0	11.7	3.0	4	5.0
12	7.7	9.0	2.75	3	3.5

The keeping quality of raw sesame oil is roughly related to its acidity. Oils with high f.f.a. have poorer keeping quality. Mere neutralization of the acidity in the cold improves the storage property of the oil. This shows that f.f.a. accelerates the absorption of oxygen by the oil. Refining of the oil, however, lowers the induction period of low acid oil and improves that of high acid oil. In order to study this effect two sets of experiments were carried out :—

A sample of high f.f.a. oil was divided into four batches. The first was the control, the second was neutralized by passing it repeatedly through a bed of quick-lime, the third was treated with animal charcoal, and the fourth was subjected to steam distillation, till it was odourless. The constants and the induction period (at 65°C.) are given in Table III :—

TABLE III.

Number.	Sample.	Acidity.	Peroxide value.	Induction period in hours.
1	Raw	7.0	1.2	3.0
2	Neutralized ...	0.05	1.1	9.0
3	Decolorized ...	6.8	1.4	3.5
4	Steam distilled ...	6.8	0.9	3.0

In the second set, three samples of oil of high, low, and average f.f.a. were saponified. The unsaponifiable matter was tested on fresh butter-fat (ghee) for its pro-oxidant property. The unsaponifiable matter was obtained from 10 g. of oil and was used with 10 g. of ghee. The results are given in Table IV :—

TABLE IV.

Oil acidity.	Peroxide value.	INDUCTION PERIOD AT 95°C. IN HOURS.		Protection factor.
		Ghee.	Ghee plus unsaponifiable matter.	
0.8	0.4	11	77	6.0
2.1	6.8	11	52	3.8
7.7	9.0	11	16	0.5

As the f.f.a. and peroxide value increase above 1 per cent, the storage property of sesame oil is reduced. In the case of low f.f.a. oil, refining process adversely affects the storage property due to inactivation of the anti-oxidant factor, while in the case of high f.f.a. oil, refining process (removal of f.f.a.) improves the storage property on account of the removal of the pro-oxidant f.f.a.

The preservation of edible oils and fats with anti-oxidant is resorted to nowadays. The effect of certain well-known anti-oxidants ethyl and propyl gallate and N.D.G.A. (American) has been tried on samples of raw sesame oil of different f.f.a. and refined oils from them. The results are shown in Table V :—

TABLE V.

The effect of anti-oxidants on sesame oil.

Number.	Acidity.	Anti-oxidant.	Concentration.	PROTECTION FACTOR AT 65°C.	
				Raw oil.	Refined oil.
1	1.1	Ethyl gallate	0.03	5	8
2	1.1	Propyl gallate	0.03	3	5
3	1.1	N.D.G.A.	0.05	7	13
4	4.0	Ethyl gallate	0.03	2	4
5	4.0	Propyl gallate	0.03	2	5
6	4.0	N.D.G.A.	0.05	5	9
7	7.0	Ethyl gallate	0.03	...	2
8	7.0	Propyl gallate	0.03	1	2
9	7.0	N.D.G.A.	0.05	3	5

N.D.G.A. is a better protective agent than ethyl or propyl gallate which are about equal in potency. With lower f.f.a. (less than 2 per cent) protection is better than higher f.f.a. (more than 4 per cent) and is double with refined oil compared to raw oil.

Part II.

Digestibility in vitro.—The hydrolysis was carried out using the experimental procedure of Weinstein and Wynne (1935-36). The experiments were carried out with raw and cooked oil. The results are given in Table VI :—

TABLE VI.

Hydrolysis of sesame oil with swine lipase (in vitro).

Fat or oil.	C.C. OF N/10 ALKALI REQUIRED AT 1 HOUR INTERVAL.										
	f.f.a.	Raw.	Fried.	Raw.	Fried.	Raw.	Fried.	Raw.	Fried.	Raw.	Fried.
Fresh cow ghee ...	0.06	2.2	2.0	4.0	3.7	6.8	6.4	8.2	7.9	10.5	9.9
Fresh oil ...	0.6	1.0	1.1	2.2	2.0	3.8	3.6	4.4	4.3	5.6	5.3
Market oil ...	1.2	1.2	1.0	2.2	2.1	3.8	3.6	4.4	4.2	5.5	5.2
	2.5	1.0	1.0	1.9	1.6	2.9	2.5	3.8	3.2	4.8	4.5
	3.4	0.8	0.9	1.8	1.5	2.7	2.4	3.5	3.2	4.4	4.0
	4.2	0.6	0.4	1.7	1.4	2.1	1.8	3.3	3.0	4.2	3.8
	5.4	0.6	0.4	1.3	1.1	1.7	1.5	2.9	3.0	3.7	3.0
	7.7	0.6	0.3	1.1	0.8	1.7	1.2	2.0	1.3	2.8	1.6

Sesame oil, like ground-nut oil, shows a decrease in the rate of hydrolysis with increase in the f.f.a. of the oil. This decrease is more marked when the f.f.a. exceeds 2 per cent and the rate of hydrolysis is decreased from the beginning unlike ground-nut oil. There is no difference in the rate of hydrolysis between raw and cooked oil. In order to find out the retarding agent the following experiments were undertaken. Sample 12 was divided into three batches: the first was the control, the second was treated with water, and the third was treated with a solution of sodium bisulphite. They were hydrolysed before and after cooking treatment. The constants and the rate of hydrolysis are given in Table VII:—

TABLE VII.

Constant.	UNTREATED OIL.		WATER-WASHED OIL.		BISULPHITE-TREATED OIL.	
	Raw.	Cooked.	Raw.	Cooked.	Raw.	Cooked.
Acidity	7.0	7.1	6.8	7.0	7.0	7.1
Peroxide value	1.2	4.0	1.2	4.2	1.3	4.3
Kriess value	15.0	16.0	10.0	12.0	1.5	2.0
Hydrolysis as c.c. of N/10 alkali in hours:						
1	0.6	0.6	0.4	0.4	1.0	1.0
2	1.1	1.0	1.5	1.3	2.0	1.9
3	1.7	1.6	2.4	2.2	3.6	3.6
4	2.0	2.0	2.7	2.4	4.0	3.8
5	2.8	2.6	3.8	3.5	5.5	5.5

The Kriess value is expressed as $R/L \times C$ where R is the tintometer reading, L length of the cell and C is the concentration of oil in gramme per c.c. of the total solution.

It is the aldehyde product that retards the digestibility of sesame oil. This accounts for the negligible difference in hydrolysis on cooking.

The effect of acidity on the inactivation of carotene in ghee in contact with sesame oil by carotene oxidase has been studied using a slight modification of Sumner and Sumner's (1940) method. The results are given in Table VIII:—

TABLE VIII.

The effect of sesame oil on the inactivation of carotene in ghee.

Sample.	Acidity.	CAROTENE CONTENT OF GHEE 0.22 MG./100 g.		
		Mg. carotene/100 g. ghee at half-hour intervals.		
		$\frac{1}{2}$	1	$1\frac{1}{2}$
Blank	0.22	0.22	0.22
Control	0.22	0.22	0.21
Fresh ...	0.6	0.21	0.20	0.19
	1.2	0.21	0.21	0.20
	2.5	0.19	0.15	0.12
Market ...	3.4	0.16	0.09	0.06
	4.2	0.11	0.07	0.05
	5.4	0.09	0.06	0.06
	7.7	0.07	0.04	0.01

High f.f.a. sesame oil accelerates the inactivation of carotene in ghee by carotene oxidase. This is similar to ground-nut oil, but the inactivation caused by sesame oil is greater than that of ground-nut oil of the same acidity.

The stability of vitamin A dissolved in sesame oil of varying acidity has been studied and the results are given in Table IX. Fresh shark-liver oil was used as the source of vitamin A. The effect of refining and cooking the oil on the stability of vitamin A has also been studied. The peroxide value of the oils was also determined.

TABLE IX.

Stability of vitamin A in sesame oil.

Acidity.	PEROXIDE VALUE OF			PERCENTAGE INACTIVATION OF VITAMIN A IN 3 HOURS.		
	Raw.	Refined.	Cooked.	Raw.	Refined.	Cooked.
0.8	0.4	0.3	0.6
1.9	1.1	0.5	4.0	16	...	20
3.0	0.3	2.0	13.0	33	10	50
5.0	11.7	2.7	14.0	50	15	62
7.7	21.0	3.0	23.0	66	15	66

The inactivation of vitamin A begins when the acidity of the oil is more than 1 per cent, and steadily increases with the acidity of the oil. Refining the oil considerably prevents the inactivation of vitamin A, while in cooked oils it is more unstable. The stability of vitamin A in sesame oil is related to its peroxide value as well.

SUMMARY.

The acidity of common sesame oil is quite high. On storage of seeds acidity rises, but in an atmosphere of nitrogen or carbon dioxide the rise is very much reduced. Acidity above 1 per cent, peroxides and heat treatment act as pro-oxidants. Anti-oxidants preferably N.D.G.A. rather than ethyl or propyl gallate help store oil better and the protection is better with lower acidity of the oil. Aldehydes present in high acid oil retard digestibility. Peroxides destroy carotene and vitamin A when sesame oil is used as solvent.

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OPIMUM ADDICTION IN ASSAM AND ATTEMPTS MADE TO ERADICATE IT.

BY

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THE use of opium by man dates back from pre-historic times although its euphoric effects were discovered only later. According to the Report of the Royal Commission on Opium (1894) opium habit was widely prevalent during the 17th century and smoking of the drug became more popular during the year 1796. From China the evil appears to have spread to the eastern part of Persia from where it found its way to India, especially to the Brahmaputra valley, through the nomadic Arabs and Mongolian tribes. The stimulating and narcotic properties of the drug coupled with the wonderful flow of ideas which occur during the early stages of smoking appealed very much to the easy-going and well-to-do sections of society in this country. The habit appears to have become common during the early period of the decline of the Moghul Empire but the use of the drug was not uncommon during the reign of Akbar as well. Had it not been for the early restrictions placed by law the evil might have spread to the length and breadth of the country. Opium-smoking dens are traceable even to-day in large towns of India but luckily the vice is confined entirely to the lower strata of society. Certain tribes, like the Kacharis, were so passionately addicted to it that they sometimes demanded to be paid in opium in lieu of money. During our survey in 1935 we found both men and women among the Kakhyens, Karens and Lpais smoking opium and smuggling the same in large quantities from the border tribal territories of China (Chopra and Chopra, 1935). The wild tribes of Turungs and Nagas also came down from hills into the valley to barter ivory for rice and opium. Certain tribes of the adjacent parts of Burma, the Parjii and Kachin, are also heavily addicted to opium. We carried out a general survey of the incidence

of this drug habit during the years 1935-38. The results of our observations are summarized in Table I :—

TABLE I.

Showing incidence of opium addiction in various districts of Assam during the year 1938-39.

District.	Annual consumption per 10,000 population in lb.
Kamrup	15·8
Goalpara	0·5
Cachar	12·0
Khasi and Jaintia hills	20·0
Naga hills	19·0
Balipara Frontier tract	58·0
Darrang	40·0
Nowgong including Mikir hills	50·0
Sibsagar including Mikir hills	80·0
Lakhimpur	118·0
Sadiya Frontier tract	178·0
Province as a whole	25·0
Standard laid down by the League of Nations (1930) for medical requirements of a country.	6 lb. per 10,000 population per annum.

It would thus be seen that the consumption of opium in all the districts except Goalpara is much higher than the standard laid down for medical purposes by the League of Nations (1930). The incidence is alarmingly high in the upper Assam valley, particularly in the tribal districts of Sadiya and Lakhimpur, where it goes up to 178 lb. and 118 lb. respectively per 10,000 population per annum. The opium conference at Simla in 1930 marked these areas as 'black spots' of the Province. The drug was mostly smoked in these areas which necessitated a much larger dosage of opium per head than when taken in the form of pill. It would further be seen that in the plains much less opium is consumed when compared with the hilly border districts. In the plain districts and in Goalpara the consumption is even lower than the League standard being only 0·5 lb. per 10,000 population per annum. The comparatively high consumption rate in Cachar is due to the fact that Northern Cachar hills are malarious and the labourers

employed in the timber industry smoke the drug under the belief that it has a prophylactic effect.

ANTI-OPIMUM CAMPAIGN OF THE FIRST CONGRESS GOVERNMENT IN 1938.

As early as 1925 the Assam Provincial Congress Committee realizing the demoralizing effects of the opium habit and its extensive prevalence among the population appointed an inquiry committee to study the problem in detail (Assam Congress Inquiry Committee's Report on Opium Habit, 1925). The available data and suggestions embodied in this Report are in an excellent work of reference. In 1938 when the first Congress Government came into power these recommendations were immediately given effect to. Having provided the necessary legislature's sanction the Provincial Government decided to stop the use and sale of the drug completely and suddenly in the three upper districts as an experimental measure, although the prohibition meant a considerable loss of revenue to the already poor and undeveloped Province of Assam. On the 1st of 'Baisakh', Hindu New Year day (13th April, 1938), one of the authors (G. S. C.) was asked to assist the Government in conducting this great experiment of prohibition hitherto not attempted elsewhere. Vigorous propaganda was carried out through the local Congress Committee, Missionary Societies and other educational institutions all over the Province. Public meetings were organized, and pamphlets and leaflets advocating the harmful effects of the opium habit were distributed freely. The evils of the habit and the necessity and the efficacy of treatment were also impressed. This helped a good deal in preparing the ground for a mass campaign to eradicate the evil.

CENSUS OF ADDICTS.

Before actually starting the campaign, a census of both the registered and unregistered addicts was carried out with the help of the local Excise and Police personnel. The unregistered addicts, i.e. those who were taking the drug through illicit means all over the area, were told that no action would be taken against them for revealing their identity. The survey we have made leads us to believe that the actual figures on the official register do not represent the exact number of addicts. There may probably be 20 per cent more addicts who were not registered but who consumed the drug through illicit sources. The percentage of registered addicts to the general population in some areas was as high as 5. The total number of addicts traced in these areas were 12,460.

MEDICAL TREATMENT CENTRES.

Special opium-addiction treatment was made available in all the Government and Tea-Garden Hospitals which were spread all over the area of prohibition. As these institutions were not considered enough to cope with the rush of addicts in a short period of 12 weeks, additional treatment centres were opened at the following centres :—

Jorhat, Tinsukhia, Ledo, Sibsagar, Dinjon, Makum, Muran, Digboi, Chabua, Dibrugarh, Marghreta and Jaipur.

Special medical staff of 40 medical men was also specially recruited and trained in the authors' method of treatment for this campaign.

ANALYTICAL STUDY OF THE RECORDS COLLECTED FROM VARIOUS
HOSPITALS AND TREATMENT CENTRES.

The compilation of records collected from the various hospitals and treatment centres after the campaign ended revealed that 10,200 persons offered themselves for the treatment within a short period of 12 weeks. It was, however, not possible for a comparatively newly trained staff to keep accounts and records accurately for scientific analysis and research purposes in such a large series but there are 8,000 cases among those who presented themselves for the treatment where reasonable data exists for analysis. These are now being published with an idea of being of some interest to the Excise and Public Health authorities of certain provinces who are experimenting with the problem of prohibition these days. Experience gained in the ætiological factors may be of some use to them.

ÆTIOLOGICAL FACTORS.

To eradicate the drug-addiction evil entirely from the life of a nation, it is of utmost importance to study the ætiological factors and the causes which lead to and promote this evil. An attempt was made to question each addict with regard to causes which lead to the habit; and other pre-disposing factors, such as general health, environment, hygiene, vocation, etc., were also recorded. The results of the data thus obtained are summarized briefly in Table II :—

TABLE II.

Showing factors responsible for the drug habit.

Casualties.	Number.	Percentage.
1. Association and example of relatives and friends	2,800	35
2. To alleviate symptom of a disease or as a cure for certain ailments ...	2,000	25
3. To overcome fatigue	1,600	20
4. For euphoric and pleasure-giving effects	1,200	15
5. To replace another addiction, such as alcohol	400	5
TOTAL ...	8,000	100

For more details the readers are referred to our previous studies (Chopra and Chopra, 1935, 1937, 1938, 1940*a*, 1940*b*).

It would thus be observed that association and example of other addicts was the main factor responsible for a larger percentage taking to the habit. Thirty-five per cent of the cases gave this as the exciting cause which made them try the drug. Disease comes next as 25 per cent took to the habit believing in its curative value for certain diseases and ailments. Fatigue was the third common cause and there were 20 per cent who took opium to overcome fatigue in a single dose towards

the evening. They stated that they felt restful and enjoyed sound sleep and were able to go to work next day fresh again. There were 15 per cent of cases in this series who stated that they indulged in the habit merely on account of its pleasure-giving and euphoric effects, the drug removed the sense of inhibition in such persons possibly by depressing the higher centres. There were a small number of individuals (5 per cent) who took it to replace the alcohol and other drug habits. It was observed that the moist and hard climate of Assam, prevalence of malaria and dysentery in the forests of Assam and lack of proper healthful recreation provided a fruitful soil for the spread of opium evil. Proper study of aetiological factor is very essential to eradicate the opium and other drug habits.

CATEGORIES OF ADDICTS.

For the purpose of treatment, the addicts were categorized into the following three groups, according to the intensity of addiction they were suffering from, which was determined by factors such as age, daily dose, duration of addiction, general health, etc. (see Table III) :—

TABLE III.

Showing grouping of 8,000 addicts treated in accordance with the intensity of addiction as calculated in respect of factors such as daily dosage, age, duration of addiction, etc.

Number treated in each group.	Percentage.	Daily dosage in grains.	Maximum age in years.
Group I 4,800	60	15 and less	40
Group II 2,400	30	16 to 30	50
Group III 800	10	31 and over	70

GROUP I: Consisted of 4,800 (60 per cent) persons of whom all were below 40 years of age and were taking the drug in doses less than 15 grains a day. The average duration of addiction in this group was shorter than in groups II and III.

GROUP II: 2,400 or 30 per cent in the series who took between 16 and 30 grains a day were classed as group II; the highest age in this group was 50 years.

GROUP III: The rest of the series, i.e. 800 or 10 per cent, fell under this group. Most of them took the drug in doses of 31 grains a day or sometimes more, and the average duration of addiction was more than in the previous two groups. The maximum age recorded was 70 years.

TREATMENT.

Lecithin and glucose treatment was carried on the same lines as previously suggested by us (Chopra, Mukherjee and Chopra, 1935) following the result of

our biochemical and clinical investigations in the Carmichael Hospital for Tropical Diseases, Calcutta, in a series of 200 cases. It was established that the lecithin and cholesterol contents of blood sera in opium addicts fell to a level below normal and that there was also a good deal of de-hydration of tissues during the withdrawal period. The present authors (Chopra and Chopra, 1935, 1937) found that, in most of the cases, the state of addiction was invariably accompanied by an increase in the fluid content of the blood, and were inclined to believe that an excessive secretion of body fluids through diarrhoea and vomiting which are common during withdrawal period will have a reverse effect, leading to an increase in the percentage of serum proteins.

The work of Chopra and Roy (1937) on the blood-lipoid changes in opium addicts before, during and after withdrawal, led Chopra and Ganguly (1939) to actually determine the effect of withdrawal on the blood fluid and also to explain, if possible, the rationale of the treatment of opium addiction with lecithin and glucose. Blood sera from the addicts under treatment were examined for total proteins before, during and after the withdrawal of the drug. In most of the cases it was found that concentration of proteins present in the serum before treatment underwent a definite increase during the withdrawal period and returned gradually almost to the pre-withdrawal value after the addict had undergone the prescribed treatment. The increase in the total protein content during the period of withdrawal, as a general rule, runs parallel to the appearance of withdrawal symptoms. Excessive outflow of water from the body causes one of the most marked withdrawal symptoms that have been observed in almost all the cases studied by us. This apparently points to a disturbance in the fluid equilibrium in general. The effect of treatment may, therefore, be taken to be to restore it to its previous level.

Chopra, Mukherjee and Chopra (*loc. cit.*) observed that increase of the euglobulin fraction in the serum of addicts probably meant an ultimate drainage of phosphate from the nerve-cells. Lecithin treatment was, therefore, suggested on that basis. In the majority of cases lecithin decreased the intensity of withdrawal symptoms and shortened their duration. In spite of its administration, the abstinence symptoms were, however, very severe in some of the subjects, and in these cases, intravenous injections of 25 cm. of 25 per cent glucose helped to ameliorate the condition. Although lecithin by itself was unable to cope with the severity of the withdrawal symptoms, it doubtless removed the craving for the drug in the majority of cases.

The rôle of glucose in coping with the abstinence symptoms can thus be understood. By treatment the ultimate effect seems to be the restoration of the water-balance. Any drug that confers a fluid-retaining power to the blood would, therefore, be expected to have good effect. Carbohydrates in general and glucose in particular are known to possess this water-retention capacity. Glucose, therefore, in addition to stocking the liver with glycogen, to enable it to cope with the unusual strain on this organ during the process of elimination of morphine, helps the retention of water in the blood and keeps the blood-fluid level at its normal value. From the above considerations we may conclude that lecithin tones up the nerves of addicts by supplying the lipo-phosphates, and glucose helps to restore the disturbed water-balance. It is, therefore, not difficult to see how these two together produce the desired effect in removing the drug-craving and alleviating the

(iv) *Insomnia*.—Over 70 per cent cases complained of insomnia on withdrawal. For mild cases no treatment was given and the patients were persuaded to sleep. Simple measures, such as hot foot-bath or a dose of bromides before retiring, were also helpful. In more severe cases the use of hypnotics, in way of soneryl, medinol, etc., had to be resorted to. Care was taken not to repeat the same drug for more than 3 consecutive days for fear that it may lead to a substitute drug habit. In most of the cases insomnia yielded to these measures and the patients felt restful and developed confidence in the efficiency of the treatment.

(v) *Gastro-intestinal disturbances*.—These manifested themselves in the way of nausea, vomiting and diarrhoea, and were more pronounced in case of group III. In milder cases sucking of ice or a few drops of adrenaline hydrochloride (1 in 1,000) solution was successful in overcoming nausea and vomiting. In a few cases of bilious vomiting, resort had to be made to stomach-wash with a soda-bicarbonate solution of 5i to a pint strength. For diarrhoea no special treatment was given except in very severe cases where bismuth and kaolin had to be administered by mouth.

The development of withdrawal symptoms was watched in each case; after the 3rd or 4th day of the treatment most of the patients reported that the discomfort had disappeared to a fair extent and exhibited a complete change in their outlook and reported that the drug had lost all its hold on them; and quite a large number stated that the very smell of the drug produced a nauseating feeling and a feeling of aversion to the use of the drug. The entire mental outlook of the addicts showed a remarkable change at the time of leaving the treatment centres. The persons who came sad and morose with the usual apathetic and pale look went with a cheerful and fresh countenance. They ate well and improved in general health and put on weight after their discharge. They became more social, self-conscious, showed an inclination to work and felt interested in their surroundings.

REHABILITATION TREATMENT.

It was not possible to properly organize the treatment during the stages of rehabilitation in the case of such a large series, specially in a comparatively backward and poor province. It was possible to arrange in a few cases only for the vocational and diversional post-withdrawal treatment which is so important in re-building the shattered personality of an addict. The individuals with a nervous diathesis and liable to suffer from a relapse were kept under observation through volunteers and relatives to prevent them from taking the drug secretly. In order to rehabilitate and train addicts who had been weaned from the drug to a new walk of life and narcotic-free environment, attempts were made to provide diversional therapy in the way of gardening and cottage industries like basket-making and mat-weaving. Some showed a relapse to the habit during rehabilitation period and the treatment had to be repeated, sometimes twice. Addition of vegetable lecithin in the form of soya beans to the diets was helpful in preventing relapses. The period of rehabilitation and re-building of personalities in certain cases sometimes extended over several months and even up to a year depending on such factors as the personality and character of the addict, the cause of addiction, the presence of a nervous diathesis, heredity, etc. It is difficult to keep from taking the drug those persons who suffered from a nervous diathesis with a

Glucose injections were also exhausted, but were locally manufactured in the Pasteur Institute, Shillong; our demands thus being met up to the end of the campaign without much difficulty.

WITHDRAWAL SYMPTOMS.

In spite of the above treatment there were a good many withdrawal symptoms to be encountered. The principal symptoms observed are detailed in Table IV:—

TABLE IV.

Showing principal withdrawal symptoms observed with their relative preponderance in the cases of the different groups.

Withdrawal symptoms observed.	Group I. 4,800	Group II. 2,400	Group III. 800
1. Pain in the body and limbs, cramps, general malaise, etc.	3,840 (80%)	2,160 (90%)	800 (100%)
2. Vaso-motor disturbances, such as sneezing, running from the eyes and nose.	2,880 (60%)	1,920 (80%)	720 (90%)
3. Cardio-vascular manifestations, such as feeble and irregular pulse, sinking sensation, cardiac embarrassment, and collapse.	960 (20%)	770 (30%)	400 (50%)
4. Insomnia	2,880 (60%)	1,920 (80%)	800 (95%)
5. Gastro-intestinal disturbances, such as nausea, vomiting and diarrhoea.	960 (20%)	720 (30%)	480 (60%)

(i) *Pain in the body and limbs, cramps, etc.*—Cramps and pain in limbs, etc., are most common and are generally due to de-hydration of the tissues resulting from loss of fluid through diarrhoea and vomiting during withdrawal. They often yield to simple measures such as massage, warm bath, etc., and may rarely require glucose and saline, A. P. C. powder or calcium-gluconate injections. Pain and cramps disappeared after 3 or 4 days of treatment.

(ii) *Vaso-motor disturbances.*—Running from the nose and eyes, sneezing, and coughing are of frequent occurrence during the first 3 days of withdrawal. No special treatment is indicated.

(iii) *Cardio-vascular symptoms.*—Cardio-vascular disturbances, mostly reflex in origin, are observed in the way of irregular pulse, sinking sensations, heaviness in cardiac region and sometimes even collapse. They are more marked in the case of persons taking higher doses and in those with longer duration of addiction. In mild cases a cup of hot tea or coffee with plenty of sugar helped in alleviating these reflex symptoms. In severe cases, cardiac stimulants, such as digitaline, cardiazole, coramine and intravenous glucose, may have to be given. In this series 6 cases died of collapse during the course of treatment. Collapse followed loss of fluid on account of persistent vomiting and diarrhoea.

It will thus be observed that 3,200 persons (40 per cent) of the addicts amongst the present series were completely relieved of the habit at the time of closure of the campaign ; 2,400 (30 per cent) succeeded in reducing their daily dose by $\frac{3}{4}$ th of the original and that 1,600 (20 per cent) showed reduction in dosage by half of their original. There were 800 (10 per cent) who showed little or no response to the treatment and amongst these 6 died during the course of treatment.

RELATION BETWEEN AGE OF ADDICTS AND RESULTS OF TREATMENT.

Table VI shows the relation between the age of the addicts and the degree of response to the treatment :—

TABLE VI.

Showing relation between the age of the addicts and the effect of treatment.

Result of treatment.	AGE OF ADDICTS.				TOTALS.
	25 years and below.	26-40 years.	41-60 years.	61 years and above.	
Complete cure ...	280	1,500	1,300	120	3,200
Dosage reduced by $\frac{3}{4}$...	180	1,030	1,040	150	2,400
Dosage reduced by $\frac{1}{2}$...	60	520	820	200	1,600
Failure of treatment ...	Nil	80	200	520	800
TOTALS ...	520	3,130	3,440	990	8,000

These results are very interesting. It will be seen that the number of persons cured in old-age groups was much less than in younger ages. Thus, out of 3,200 cases who were completely cured, 1,780 were below 40 years of age, 1,300 between 41 and 60 and only 120 persons were above 60 years of age. In this series there were 520 persons below 25 and out of these 280 got rid of the habit completely. There were 3,130 persons between 26 and 40. Amongst these 1,500 were completely cured. There were 3,440 persons between 41 and 60. Amongst these 1,300 were completely cured of the evil. Amongst the 990 aged above 61 years only 120 could get rid of the habit completely.

Similarly, reduction of dosage was also more marked in the younger cases. Conversely, failure of treatment was more marked in the case of higher age groups. It will be observed that in the 26-40 age group consisting of 3,130 cases, treatment failed in 80 cases only ; in the 41-60 age group of 3,440 cases the treatment failed in 200 cases, while in 990 persons aged above 61, treatment failed in 520 cases, i.e. in more than 60 per cent. Thus, the younger persons were more amenable to the treatment than the older and more confirmed addicts.

RELATION BETWEEN DOSAGE AND TREATMENT.

The effects of the dosage on treatment have been analysed in Table VII :—

TABLE VII.

Showing relation between dosage and treatment.

Result of treatment.	Dose in grains :			TOTALS.
	15 and under.	16-30	31 and over.	
Complete cure effected ...	1,200	1,150	850	3,200
Dose reduced by 3/4 ...	1,020	750	630	2,400
Dose reduced by 1/2 ...	525	325	750	1,600
Failure ...	98	200	502	800
TOTALS ...	2,843	2,425	2,732	8,000

It will be seen that cures were more marked in the case of smaller doses. Out of 2,843 persons who took the drug in doses under 15 grains a day as many as 1,200 showed complete cure as compared with 750 out of 2,425 cases who took between 16 and 30 grains, and 1,150 out of 2,732 persons who took 31 grains and more a day. The reduction in dosage was also well marked in case of small-dose group. Failures were more conspicuous amongst higher doses. Thus, the results were very encouraging in persons who took below 31 grains of the drug a day.

DURATION OF ADDICTION AND RESULTS OF TREATMENT.

These were also studied and the observations are recorded in Table VIII :—

TABLE VIII.

Showing results of treatment and duration of addiction.

Result of treatment.	DURATION OF ADDICTION IN YEARS.			TOTALS.
	10 years and under.	11-20 years.	21 years and over.	
Complete cure effected ...	2,585	615	10	3,200
Dosage reduced by 3/4 ...	315	2,065	20	2,400
Dosage reduced by 1/2 ...	800	710	90	1,600
Failure ...	200	470	130	800
TOTALS ...	3,900	3,850	250	8,000

It will be seen that cures were more marked in cases of shorter duration of addiction, viz. 10 years and under where out of 3,900 persons 2,585 completely responded to the treatment as compared with 615 out of 3,850 with 11-20 years' duration and only 10 out of 250 with 21 years and over duration. Conversely again, the failures of treatment were more marked in the case of larger-duration addicts, but with above 21 years out of 250 persons 130 showed no response to the treatment.

SUMMARY.

The authors have tried to summarize the results of the first large mass-scale experiment in total prohibition of opium tried 10 years ago by the Congress Ministry. Over ten thousand confirmed opium addicts offered themselves for the treatment within a short period of 12 weeks. The results discussed in the foregoing pages prove that the lecithin and glucose treatment is suitable for mass application in areas intensely addicted to opium habit.

The authors are grateful to the Hon'ble Premier Shri Gopinath Bardoli who was keenly interested in the problem and remained most of the time with us and encouraged us with his valuable advice at all stages, and also to Dr. K. C. K. E. Raja, the Director-General of Health Services, New Delhi who very kindly went through the data and gave his valuable advice and help and encouraged us to publish this work. Our thanks are also due to Rao Bahadur D. Sharma, Excise Commissioner, and to Colonel Hesterlow and their staff who worked with us night and day with unfailing zeal.

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FOLIC ACID IN THE TREATMENT OF MACROCYTIC ANÆMIA IN PREGNANCY.

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WITH A STATISTICAL NOTE

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WORKERS in America and in Europe refer to macrocytic anæmia during pregnancy and in puerperium as pernicious anæmia of pregnancy, though they differentiate it, and rightly too, from Addisonian pernicious anæmia, as many of the criteria of true Addisonian anæmia are not found in this. To avoid any confusion between the two conditions, we strongly feel that the term pernicious anæmia should be confined to cases of Addisonian pernicious anæmia only, and that the term macrocytic anæmia in pregnancy should be used for all other cases of macrocytic anæmias seen in a pregnant woman or in the puerperium, as we are of opinion that anæmia seen in the puerperium is only a continuation of the anæmia which developed during pregnancy. Probably on these grounds, Davidson, Davis and Innes (1942) suggested the term 'megalocytic anæmia of pregnancy' for these cases but later Davidson, Girdwood and Clark (1948) are found again to call these cases as 'pernicious anæmia of pregnancy and puerperium'.

In Europe and America the incidence of anæmia in pregnancy is very low and as a cause of maternal mortality, anæmia in pregnancy is almost negligible in those

countries (Davidson *et al.*, 1948). In India, however, anaemia occupies a very high place in the list of causes of maternal mortality and morbidity—in many places it is second in list and in some it is even the first (Napier and Neal-Edwards, 1942). From recent observations, it appears that the incidence of anaemia in pregnant women in India is still high not only in the poorer class of people but in the middle class of people as well, probably on account of lack of even the minimum amount of animal protein in the diet due to non-availability and very high price of the protein-containing foodstuffs. Anaemia in most of these cases falls under the category of 'deficiency dyshaemopoietic anaemia' and is macrocytic in type. Encouraged by our results with 'folic acid' in cases of nutritional macrocytic anaemia in the general population (Das Gupta and Chatterjea, 1946), we undertook the study of its effect in cases of macrocytic anaemia in pregnancy, the haemogram and marrowgram of which are indistinguishable from the cases of nutritional macrocytic anaemia. Another reason for undertaking this work is the paucity of reports on the effect of 'folic acid' in cases of anaemia in pregnancy.

Selection of cases.—Cases of grave anaemia either during pregnancy or within a few days of confinement were selected; many of the cases had previously a few injections of liver extracts of unknown potency prior to folic-acid therapy and some of them had transfusion of blood as well. The red cell count in most of the cases was in the neighbourhood of one million and only in a few cases this was above 1.5 million. The anaemia was generally macrocytic in type though in a few cases the mean corpuscular haemoglobin concentration was low, denoting dimorphic nature of the anaemia. Sternal puncture was done on many of the cases, the bone-marrow in all the cases was cellular and in most of the cases the marrow was megaloblastic. The cases will be considered under two main groups:—

GROUP A: Cases where the treatment was given during pregnancy.

GROUP B: Cases where the treatment was given within a few days after confinement or started just a day or two before confinement.

Method of study.—Treatment with folic acid was started without waiting to find out the effect of hospitalization as many of the cases were very anaemic and also on account of the difficulty of keeping the patients over a long period. In most cases examination of the blood was supplemented by examination of the bone-marrow material obtained by sternal biopsy; in describing the immature cells of the red cell series, Israel's (1939) nomenclature was followed. Reticulocyte counts were done from the 4th to about the 10th day of folic-acid treatment and detailed examination of the blood was carried out about once a week.

Method of administration and dose.—Folic acid (Folvite tablets, Lederle) was allways given orally in doses of 30 mg., 20 mg. and 10 mg. a day. All the cases excepting 3, 1 in group A and 2 in group B, were treated with folic acid and except in 2 cases in group B folic acid was the only haematinic given at any time.

RESULTS.

GROUP A: There were 18 cases in this group. Of these 3 patients died, 1 on the 2nd day, 1 on the 3rd day and 1 on the 8th day after folic-acid treatment—in the last case examination of the blood on the 7th day showed some improvement

in the blood picture though the patient died the next day. One patient left hospital on the 10th day of treatment; this patient showed clinical improvement but a second blood count was not done before she left the hospital. The results of treatment in the remaining 14 cases along with the relevant data are shown in Table I and Charts in the P series (see Plate IV).

GROUP B: There were 27 cases in this group. Of these 5 died, 1 on the same day, 3 on the 2nd day and 1 on the 17th day from an attack of pneumonia; this last patient had shown excellent response both clinically and hæmatologically within the short period. Five patients left the hospital during the course of treatment, 1 on the 4th day, 1 on the 6th day, 2 on the 9th day and 1 on the 10th day of treatment. A second blood count was done on 2 patients who went away on the 9th as also on the patient who went on the 10th day and all the 3 cases showed moderate improvement in their blood pictures. The results of treatment in the remaining 17 cases together with the relevant data are shown in Table II and Charts in the C series (see Plate V).

ANALYSIS OF THE DATA OF THE CASES SHOWN IN TABLES I AND II.

Hæmogram.—The blood picture was dimorphic in type in 9 cases: in 4 cases in group A (Nos. 11, 12, 13 and 14) and in 5 cases in group B (Nos. 13, 14, 15, 16 and 17). The blood picture in the remaining 22 cases was macrocytic in type and simulated the blood picture of cases of nutritional macrocytic anaemia.

Marrowgram.—**GROUP A:** Sternal puncture was done in 11 cases and in all these cases the marrow was found to be megaloblastic, either frankly or slightly; details are given in Table I.

GROUP B: Sternal puncture was done in 12 cases: in 3 cases the marrow was normoblastic and in the remaining 9 cases the marrow was megaloblastic, either frankly or slightly; details are given in Table II.

Reticulocytosis.—The reticulocyte response was denoted as excellent if the peak reached the expected height according to the Riddle chart; good if the peak was about 75 per cent, and fair if it was about 50 per cent of the expected height.

GROUP A: Reticulocytosis was excellent in 3 cases (Nos. 2, 6 and 12), good in 2 cases (Nos. 4 and 7) and fair in 4 cases (Nos. 1, 9, 11 and 13).

GROUP B: Reticulocytosis was excellent in only 1 case (No. 1), good in 4 cases (Nos. 2, 7, 12 and 13) and fair in 6 cases (Nos. 5, 6, 9a, 10, 14 and 15).

RATE OF IMPROVEMENT OF THE RED CELLS PER DAY FOR THE FIRST TWO WEEKS.

According to the formula of Riddle $1 = 0.93 - 0.214 \text{ EO}$; where 1 is the improvement rate and EO is the erythrocyte count before therapy (Della Vida and Dyke, 1942).

GROUP A: The red cell count increased up to about the expected range in 2 cases (Nos. 3 and 12), to about 75 per cent in 5 cases (Nos. 1, 2, 4, 5 and 8), to about 50 per cent in 5 cases (Nos. 6, 7, 9, 11 and 14) and less than 50 per cent

of the expected rise in 2 cases (Nos. 10 and 13). In case No. 8, with the increase of the dose from 10 mg. to 30 mg., the improvement rate increased from about 75 to 100 per cent.

GROUP B: Increase of red cells up to about the expected range was noted in 6 cases (Nos. 1, 2, 4, 5, 7 and 13), up to about 75 per cent in 3 cases (Nos. 6, 11 and 14), up to about 50 per cent in 1 case (No. 3), increase less than 50 per cent in 2 cases (Nos. 8 and 10) and only slight improvement in 3 cases (Nos. 9, 15 and 16). With increase of dose from 10 mg. to 30 mg. case No. 9 showed increased rate of improvement.

FINAL IMPROVEMENT.

GROUP A: The improvement was very good in only 1 case (No. 1); good in 8 cases (Nos. 2, 3, 4, 5, 7, 8, 9 and 11) and fair in 3 cases (Nos. 6, 12 and 13).

GROUP B: Very good improvement was noted in 4 cases (Nos. 1, 2, 3 and 5), in Nos. 1 and 2 after 30 mg. and in Nos. 3 and 5 after 20 mg. Good improvement was noted in 3 cases (Nos. 4, 7 and 13). The improvement was fair in 6 cases (Nos. 6, 8, 10, 11, 14 and 16). Case No. 16 was also given ferrous sulphate along with folic acid.

CORRELATION BETWEEN THE VARIOUS FACTORS.

(i) *Bone-marrow reaction with other factors.*—In cases with megaloblastic bone-marrow, there does not appear to be any correlation of the number of megaloblasts in the marrow with the reticulocyte response, or with the rate of improvement of red cells or with the final improvement. Of the 3 cases with normoblastic marrow in group B, one case (No. 4) with 20 mg. showed good final improvement which was preceded by very good improvement rate in the red cell count in the initial stage but was attended with only moderate reticulocytosis. A second case (No. 15) showed fair reticulocyte response but both the rate of improvement and the final improvement were very poor; this patient later improved on iron.

(ii) *Reticulocytes with other factors.*—In either group, no correlation is seen between the reticulocyte response and the dose of folic acid and/or the reaction of the marrow; neither is any correlation seen between the reticulocyte response and the rate of improvement of the red cells and/or the final improvement.

(iii) *Initial rate of improvement of red cells with other factors.*—In many cases the initial daily improvement rate during the first two weeks was not a true index of the final improvement, nor was there any correlation between the initial improvement and the reticulocyte response. The improvement rate did not seem to depend on the percentage of megaloblasts in the marrow, but the improvement rate appeared to be poorer with 10-mg. dose than with 20-mg. or 30-mg. doses.

(iv) *Dose of folic acid with other factors.*—In both the groups, the patient receiving 10 mg. a day showed poorer response in almost all aspects as compared to those receiving 20 mg. or 30 mg. a day. In a few cases (No. 8 in group A and No. 9 in group B), the response was even found to be enhanced by increase of the dose from 10 mg. to 30 mg. There does not, however, appear to be any great difference in response in the different aspects between 20-mg. and 30-mg. doses.

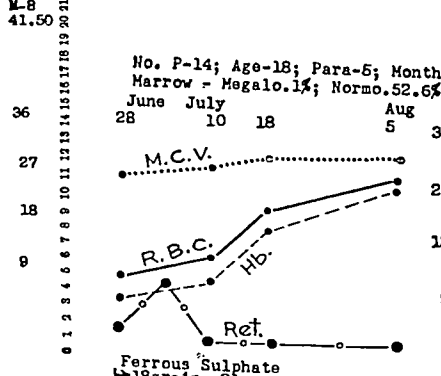
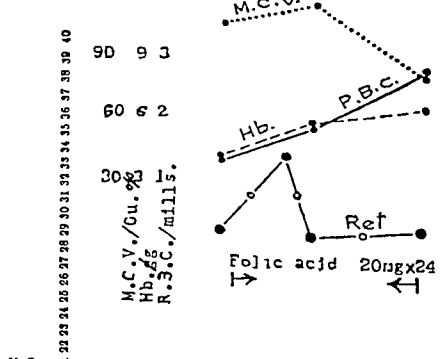
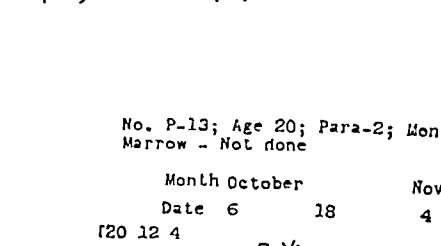
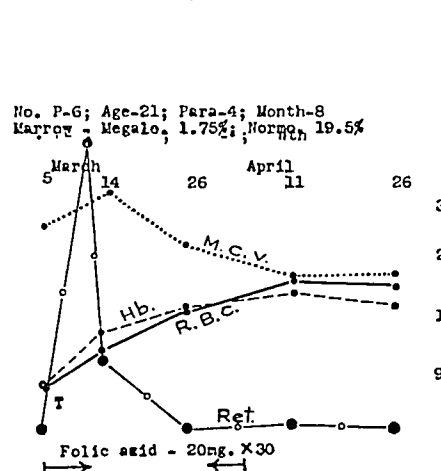
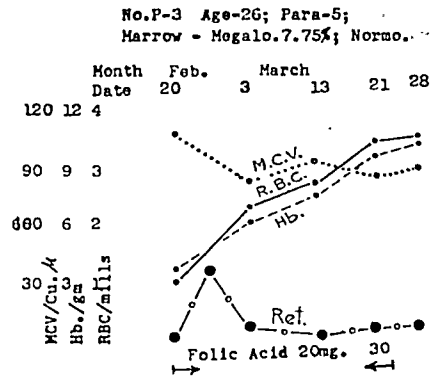
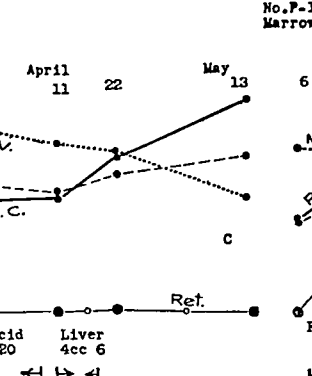
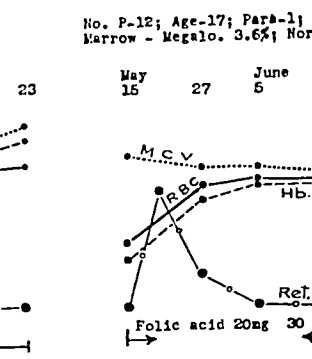
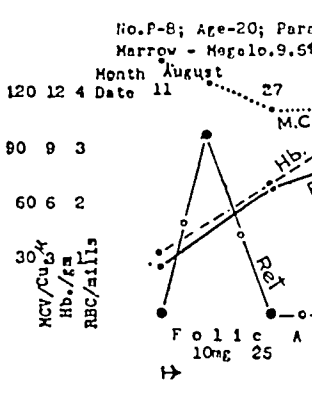
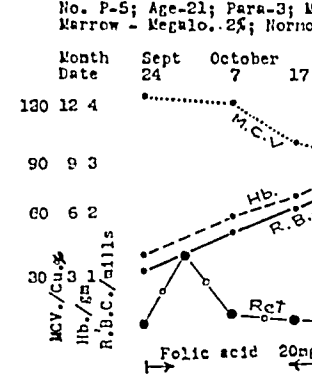
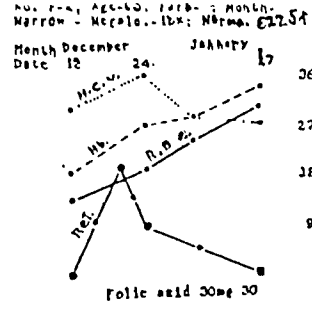
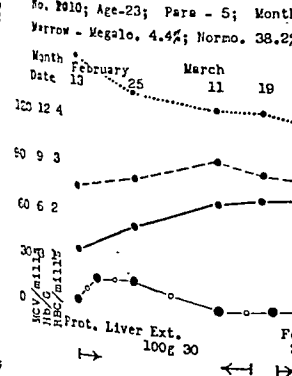
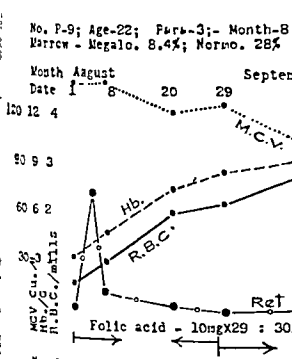
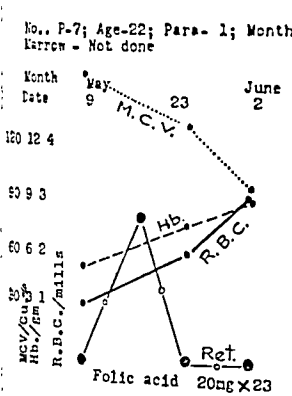
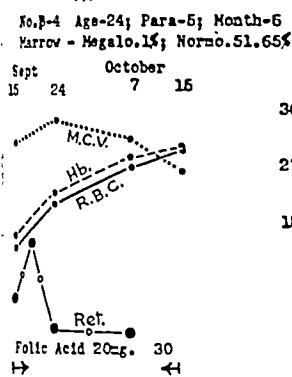
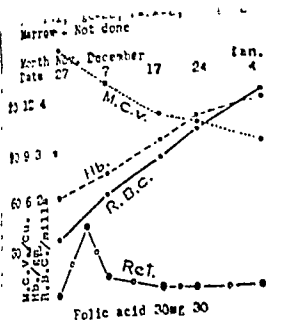
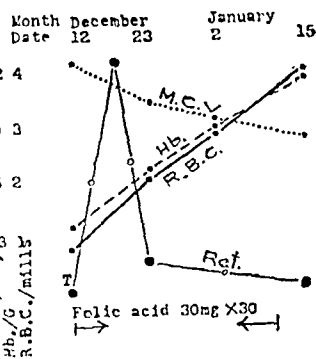
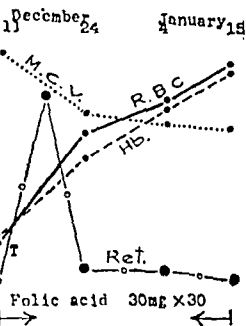


PLATE V.

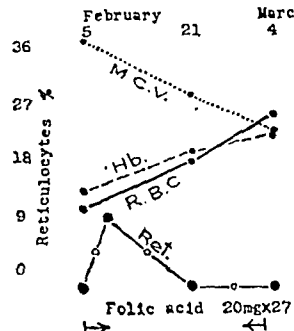
No. C-1; Age-28; Para - 1
Marrow - Megalo. 9.25%; Normo. 10.5%



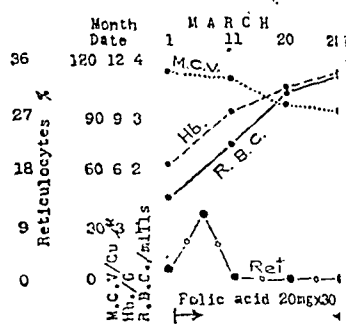
No. C-2; Age - 22; Para - 1
Marrow - Megalo. 14.75%; Normo. 22.5%



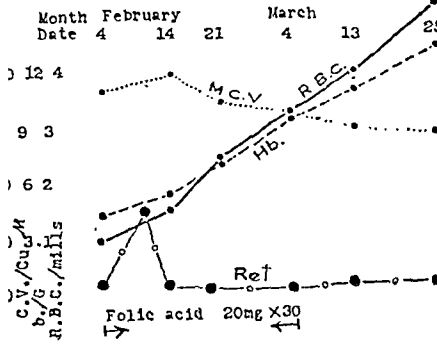
No. C-4; Age - 22; Para - 3
Marrow - Megalo. 0; Normo. 35.2%



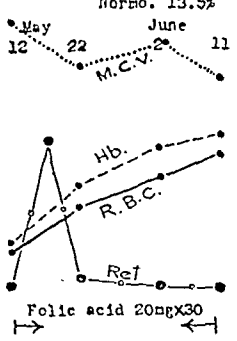
No. C-5; Age-18; Para-1
Marrow - Megalo. 0.8%; Normo. 54.8%



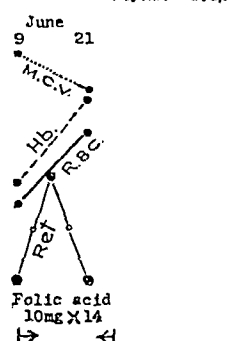
No. C-3; Age - 20; Para - 2
Marrow - Megalo. 1.6%; Normo. 35.2%



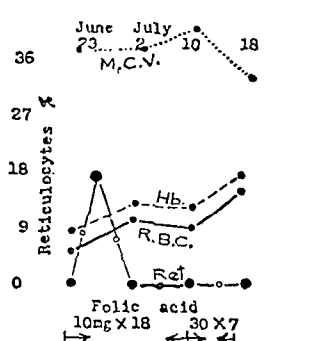
No. C-6; Age-20; Para-2
Marrow - Megalo. 7.5%; Normo. 13.5%



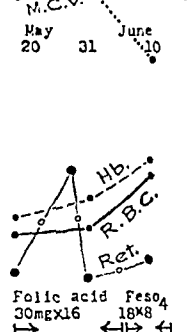
No. C-7; Age-22; Para-2
Marrow - Megalo. 1.4%; Normo. 45.6%



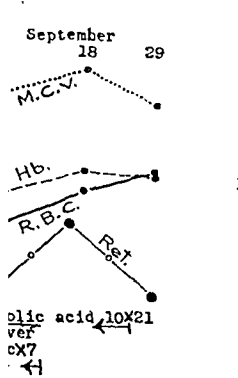
No. C-9; Age-20; Para-8
Marrow - Megalo. Not done



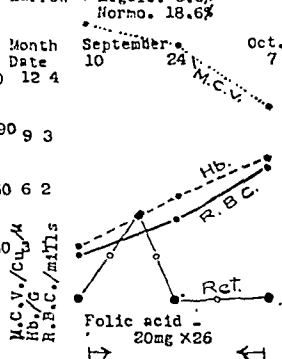
No. C-15; Age-25; Para-1
Marrow - Megalo. 0.8%; Normo. 32.2%



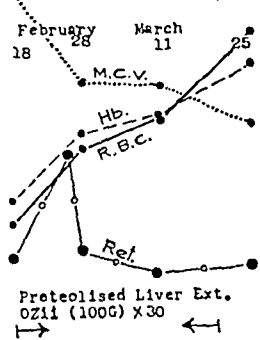
No. C-8; Age-34; Para-8
Marrow - Not done



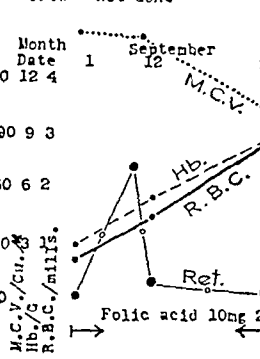
No. C-10; Age-26; Para-2
Marrow - Megalo. 5.6%; Normo. 18.6%



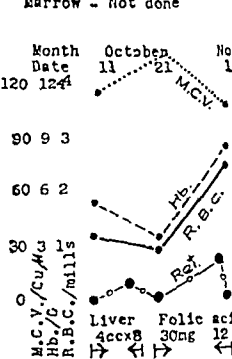
No. C-12; Age-38; Para-8
Marrow - Megalo. 13.4%; Normo. 36.6%



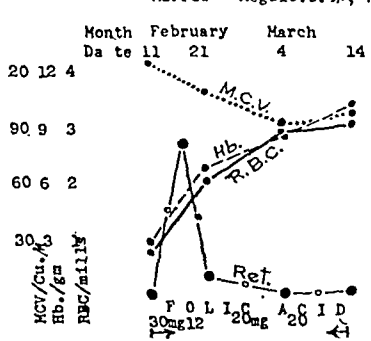
No. C-14; Age-30; Para-7
Marrow - Not done



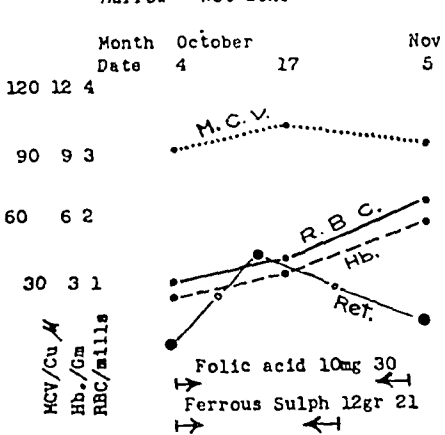
No. C-11; Age-16; Para-1
Marrow - Not done



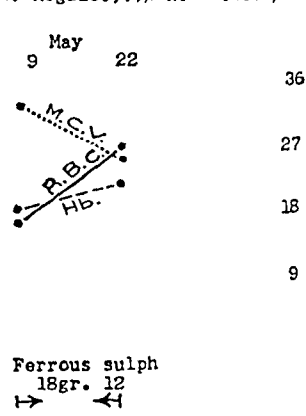
No. C-13; Age- Para-2
Marrow - Megalo. 0.4%; Normo. 49.4%



No. C-16; Age-19; Para-2
Marrow - Not done



No. C-17; Age-25; Para-4
Marrow: Megalo. 0.0%; Normo. 48.6%



(v) *Final improvement with other factors.*—In both the groups final improvement was found to be definitely better with 30-mg. and 20-mg. doses than with 10-mg. dose, but there does not seem to be any correlation with other factors, viz. reaction of the marrow, reticulocyte response and initial rate of increase of red cells.

Relative efficacy of folic acid and other hæmatinics in the different type.—One case (No. 10 in group A) did not show any improvement with folic acid though she showed some improvement with proteolysed liver extract given before folic acid and later showed very good improvement with liver extract given parenterally. A case of dimorphic anaemia (No. 14 in this group) with slight megaloblastic reaction of the marrow showed good improvement with iron—folic acid was not given to this case.

In group B, 1 case (No. 12), with frankly megaloblastic reaction of the marrow, showed excellent improvement with proteolysed liver extract given by mouth—the rate of improvement of the red cells as also the final improvement were perhaps the best in the whole series. A case of dimorphic anaemia with normoblastic marrow (No. 15) showed very little improvement with folic acid but showed better improvement with ferrous sulphate given after folic acid. A second case of dimorphic anaemia (No. 16) showed moderate improvement with combined treatment with folic acid and ferrous sulphate, while a third case (No. 17) with normoblastic marrow showed good improvement with ferrous sulphate alone.

DISCUSSION.

The results of our present study indicate that synthetic folic acid (Lederle) is a potent hæmatinic in the treatment of macrocytic anaemia in pregnancy. The results also indicate that cases of dimorphic anaemia do not respond so well to folic acid as do the cases of frankly macrocytic anaemia resembling the cases of nutritional macrocytic anaemia in the non-pregnant females and in the males. It will also be noted that the response to folic acid in the cases of normoblastic marrow was poorer than what was generally seen in cases with megaloblastic marrow. Exception, however, was seen in 1 case (No. 4 of group B). This case showed quite good response to folic acid though the reaction of the marrow was normoblastic.

The examination of our data in Tables I to IV shows that in the cases under review the minimal optimal dose must be nothing less than 20 mg. a day, and some of the cases may even require 30 mg. a day. It may be mentioned here that it has been our experience that the dose of liver extract required to get the best results in the treatment of severe cases of macrocytic anaemia in pregnancy is much bigger than what is usually required for the treatment of uncomplicated cases of nutritional macrocytic anaemia. The increased dose of hæmatinic required in these cases may be explained on the following grounds: During pregnancy there is always an increased demand for the erythrocyte maturing factor to meet the extra requirements of the foetus, which, without any consideration about the source of supply, continues to draw its full quota mercilessly from the mother. This together with the diminished intake of food, as is usually the case with these patients, may so deplete the reserve that to get any appreciable response the hæmatinic must be supplied in much bigger doses than what are usually required.

in the treatment of uncomplicated cases of nutritional macrocytic anaemia. Probably on these grounds, Davidson *et al.* (1948) used 10 mg. a day, after initial dose of 20 mg. to 30 mg. for a few days, in treating the cases of pernicious anaemia of pregnancy, though Davidson and Girdwood (1947) used only 5 mg. of folic acid per day in other cases and strongly criticized the use of folic acid in doses of over 5 mg. a day. Even then the response they obtained was only fair in comparison to the response that we obtained with bigger doses. On the other hand, excellent results were obtained by Moore *et al.* (1945) by using 20 mg. a day intravenously and recently by Benjamin (1948) by using a very big dose, e.g. 135 mg. a day. We, however, feel that the use of such big doses is not at all justified, specially on account of the limited availability of the drug and its high cost. We are, therefore, strongly of the opinion that in the treatment of macrocytic anaemia in pregnancy folic acid must be given in doses of 30 mg. a day to start with, which, however, may be brought down to 20 mg. or even 10 mg. a day after 10 or 15 days. From our experience we are also of the opinion that treatment with folic acid should generally be continued for 30 days, which is usually sufficient to improve the blood picture considerably and in some cases may even bring it to about the normal level. In cases where the treatment is given during pregnancy, after the first course of treatment with satisfactory results, it is desirable that the treatment with small dose of folic acid, e.g. 5 mg. a day, should be continued till the termination of pregnancy. It is needless to add that in these cases the examination of blood must be carried out periodically and, if the blood values show any tendency to fall at any time, the dose of folic acid should at once be increased.

The reticulocyte response was high in many cases and was generally much higher than what is usually seen after potent liver extracts. In most cases the initial reticulocyte response did not, however, give any accurate indication of the final improvement. A correct indication about the final improvement was also not obtained by observing the rate of initial red cell increase according to the formula of Della Vida and Dyke (*loc. cit.*). In other words, there did not appear to be any strict correlation between the initial haemopoietic response as shown by reticulocytosis and the rate at which the red cells increase during the first two weeks with the final improvement.

Though folic acid was given to majority of the cases in the series, liver extracts, parenteral or oral, were tried in a few cases of macrocytic anaemia and ferrous sulphate in a few cases of dimorphic anaemia. It will be noted that in the frankly macrocytic anaemias with megaloblastic marrow, the results obtained with liver preparations were as good as those obtained with folic acid as in case No. 12 of group B and in case No. 10 of group A. In cases showing evidence of dual deficiency, the dimorphic anaemias, folic acid is not as effective as in the cases of macrocytic anaemias with megaloblastic marrow. On the contrary it will be noted that in some of the cases, e.g. No. 14 of group A and No. 17 of group B, the improvement noted with ferrous sulphate alone was much better than what was noted with folic acid alone. It will also be noted that all cases of macrocytic anaemias with megaloblastic reaction of the marrow do not react to folic acid, e.g. case No. 18 of group B. This patient failed to react to folic acid but reacted well to liver extract. A contrast is provided by patient No. 11 of group A which did not react to liver extract but reacted well to folic acid.

SUMMARY.

1. Synthetic folic acid (Lederle) is a potent hæmatinic in the treatment of macrocytic anæmia in pregnancy with megaloblastic marrow.

2. It should be given in doses of 20 mg. to 30 mg. a day.

(i) In the pregnant cases, the treatment should be given for 30 days in the first instance, which generally helps to bring the blood picture to about the normal level, and then continued with 5 mg. a day till the termination of pregnancy.

(ii) In cases where treatment is given after confinement, the therapy should be given for about 30 days which is usually sufficient to bring the blood picture to about the normal level.

3. Not all the cases of macrocytic anæmia with megaloblastic marrow react to folic acid though they may not really be refractory cases as they respond to liver given subsequently ; on the other hand, cases not responding to liver were found to respond well to folic acid.

4. In some cases the response to treatment with liver extracts, given parenterally or orally as proteolysed liver extracts, was found to be quite as good as seen after folic acid.

5. The cases of dimorphic anæmia do not react very well to folic acid.

CONCLUSION.

Folic acid in doses of 20 mg. to 30 mg. a day is a potent hæmatinic in the treatment of macrocytic anæmia in pregnancy, but it has got its limitations. To get the best results, it is imperative that before instituting treatment with folic acid, which is fairly expensive and is not easily available in India, the blood and if possible the bone-marrow of the patient must be carefully examined according to modern hæmatological technique to find out the suitability of the case.

This work was carried out mainly on patients in the Eden Hospitals, Calcutta, under the care of Professor M. N. Sarkar, B.A., M.B., F.R.C.S., F.R.C.O.G., Professor G. B. W. Fisher, F.R.C.S., M.R.C.O.G., and Professor S. Bose, B.Sc., M.B., F.R.C.S., M.R.C.O.G., and a few cases in the Campbell Hospitals under the care of Dr. C. Mukherji. Our grateful thanks are due to all these officers and their house-staff for their active co-operation. Our thanks are also due to Mr. S. Ghose, Research Assistant, Hæmatological Unit, I.R.F.A., and to Mr. A. K. Biswas, Laboratory Assistant, Endowment Fund, for technical assistance. We are thankful to Messrs. Lederle Laboratories Division, American Cyanide Company, New York, N.Y., for the supply of Folvite tablets.

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STATISTICAL NOTE.

BY

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ACCORDING to Riddle (1930, 1940), a level of 4.5 millions red cell count attained at the end of a treatment lasting for a period of 6 to 8 weeks in the case of pernicious anaemia is adequate to accept the potency of a hæmatinic. This test is inapplicable in the present instance, as in none of the cases the period for which the treatment was continued extended to more than a month. Considering, however, the improvement in the final reading of the red cell count, there is every reason to expect that the standard prescribed by Riddle would have been reached in many of the cases tried by the folic-acid treatment if it had been continued sufficiently long. The reticulocyte response is admittedly an imperfect criterion for measuring the standard of the hæmatinic potency and so also is the initial rate of increase of red cell count during the first two weeks. In the present study it is observed that the correlation between the initial rate of increase and the final improvement ($r = 0.47$, $n = 13$ for series A; $r = 0.13$, $n = 13$ for series B) is not significant and therefore the initial rate of increase is not an adequate index of the final improvement. For the above reasons, the rate of increase per day in the red cell count during the entire period of treatment is taken as the criterion for testing the effect of the treatment.

Comparing the effect of dose 20 mg. and dose 30 mg. on the mean increase in red cell count it is found that there is no significant difference between the two doses (value of $t = 1.02$, for $n = 21$). The difference between the effect of dose 30 mg. and dose 10 mg. does not attain statistical significance when the whole data are considered but when the only normoblastic case of the group is removed the difference becomes significant (value of $t = 1.73$ in the first case and 2.32 in the second case for 14 and 13 degrees of freedom respectively).

There is no significant relationship between reticulocyte peak and the dose of folic acid used, as may be seen from the results of the analysis of variance given below :—

Variance between doses = 20.5 with 2 degrees of freedom.

Variance within doses = 82.3 with 24 degrees of freedom.

Variance ratio = 0.2.

In order to test whether cases with varying number of megaloblasts react differently to folic-acid treatment, the correlation between the number of megaloblasts and the change in the blood picture per day, in the three characters, viz.

red cell count, hæmoglobin and mean corpuscular value, has been worked out for the two series separately and tabulated below. Similar correlation of the change in blood picture with the reticulocyte peak is also given in the same Table. None of the correlation coefficients is significant :—

TABLE.

Values of correlation between number of megaloblasts and reticulocytes with the change in r.b.c., Hb. and m.c.v.

		Increase in r.b.c. per day.	Increase in Hb. per day.	Decrease in m.c.v. per day.
Number of megaloblasts in marrow	{ A	0·37	0·42	0·21
	{ B	— 0·01	— 0·10	— 0·23
Reticulocyte peak ...	{ A	— 0·04	— 0·20	0·42
	{ B	0·09	0·40	0·33

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TABLE I.
Cases treated during pregnancy—group A.

				BEFORE TREATMENT.				MARROWGRAM.				TREATMENT.	
Number.	Age.	Para.	Month.	HEMOGRAM.			M.c.h.c. per cent.	Total nucleated cells per c.mm.	Megalo. per cent.	Normo. per cent.	Hæmatinic.	Dose.	Days.
				Hb. in g.	R.b.c. in mills.	M.c.v. in cu. μ .							
1	22	2	8	6.18	1.26	158.7	30.9	Folic acid	30 mg.	30
2	35	6	9	6.60	1.65	115.2	34.7	270,000	15.0	32.25	"	30 mg.	30
3	26	5	8	3.71	1.91	108.9	33.7	Clotted-cellular*	7.75	62.25	"	20 mg.	30
4	24	5	6	5.22	1.55	103.2	32.6	214,000	1.0	51.60	"	20 mg.	30
5	21	3	8	4.12	1.11	126.1	29.4	45,000	2.0	53.20	"	20 mg.	30
6	21	4	8	2.75	0.84	111.9	30.5	Cellular*	1.75	19.50	"	20 mg.	30
7	22	1	8	5.22	1.13	159.3	29.2	"	20 mg.	23
8	20	4	6	3.48	0.85	144.4	29.4	Cellular*	9.6	20.80	"	10 mg.	23
9	22	3	8	9.46	2.08	113.9	30.7	110,000	8.4	28.0	"	30 mg.	12
				3.45	0.65	138.4	38.3				"	10 mg.	29
				8.70	2.20	125.0	31.6				"	30 mg.	23
10	23	5	8	7.56	1.25	160.0	37.8	150,000	4.4	38.2	Prot. liv. ext.	100 g.	30
				8.66	2.34	126.1	29.4				Folic acid	20 mg.	20
				7.56	2.37	105.4	30.1				Liver ext.	4 e.c.	6
11	25	4	8	5.50	1.85	100.1	29.7	108,000	1.25	41.5	Folic acid	20 mg.	17
12	17	1	8	3.80	1.34	104.4	27.5	140,000	3.60	27.0	"	20 mg.	30
13	20	2	9	4.06	1.33	105.2	29.0	"	20 mg.	24
14	18	5	4	2.61	1.27	92.4	21.7	152,000	1.0	52.6	FeSO ₄	18 gr.	21

* About 100,000 per c.mm.

TABLE I—*concl.*

Number.	Age.	Para.	Month.	AFTER TREATMENT.					RETICULOCYTE PEAK.		RATE OF INCREASE OF RED CELLS PER WEEK FOR FIRST TWO WEEKS.	
				HEMOGRAM.					Actual.	Expected.*	Actual.	Expected.†
				Hb. in g.	R.b.c. in mills.	M.c.v. in cu. μ .	M.c.h.c. per cent.	Interval between two counts (days).				
1	22	2	8	11.68	4.09	90.5	31.8	38	13.0	27.0	0.609	0.660
2	35	6	9	11.68	3.58	94.9	34.3	35	20.8	19.0	0.413	0.570
3	26	5	8	10.17	3.51	89.7	32.0	36	11.0	34.0	0.834	0.713
4	24	5	6	10.10	3.35	89.5	33.6	30	15.0	21.0	0.546	0.590
5	21	3	8	9.70	3.26	85.8	34.6	41	12.6	31.0	0.476	0.690
6	21	4	8	7.42	2.69	83.2	32.9	37	48.9	40.0	0.490	0.750
7	22	1	8	8.86	3.00	96.6	30.5	23	24.0	30.0	0.437	0.680
8	20	4	6	9.46	2.68	113.9	30.7	25	29.8	40.0	0.645	0.750
9	22	3	8	11.65	3.50	102.0	30.8	12	0.476	0.356
				8.70	2.20	125.0	31.6	30	22.6	46.0	0.420	0.790
				10.40	2.94	112.3	35.6	24	0.210	0.450
				8.66	2.34	126.1	29.4	30	6.9	27.0	0.294	0.662
10	23	5	8	7.56	2.37	105.4	30.1	14	3.6	10.0
				9.62	4.38	70.7	31.0	30	0.435	0.438
11	25	4	8	7.68	2.82	97.5	27.9	20	10.1	16.0	0.336	0.534
12	17	1	8	7.50	2.70	91.2	30.1	30	22.6	25.0	0.679	0.640
13	20	2	9	6.38	1.83	80.8	29.0	27	12.6	25.0	0.280	0.645
14	18	5	4	9.86	3.12	105.7	29.8	38	10.0	27.0	0.420	0.658

* According to Riddle in pernicious anæmia.

† According to Della Vida and Dyke.

TABLE II.
Cases treated after confinement—group B.

Number.	Age.	Para.	BEFORE TREATMENT.					TREATMENT.			
			HÆMOGRAM.			MARROWGRAM.		Hæmatinic.	Dose.	Days.	
			Hb. in g.	R.b.c. in mlla.	M.c.v. in cu. μ .	M.c.h.c. per cent.	Total nucleated cells per c.mm.	Megalo. per cent.	Normo. per cent.		
1	28	1	3.7	0.88	125.0	33.7	Clotted-cellular*	9.25	10.5	30	30 mg.
2	22	1	2.75	0.72	125.2	30.6	Cellular*	14.75	22.5	30	30 mg.
3	20	2	4.4	1.02	118.2	35.2	130,000	1.6	35.2	30	20 mg.
4	22	3	5.7	1.52	131.5	28.8	378,000	0.0	35.2	27	20 mg.
5	18	1	6.32	1.64	115.8	33.2	Cellular*	0.8	54.8	30	20 mg.
6	20	2	2.88	0.75	146.6	26.2	150,000	7.5	13.5	30	20 mg.
7	22	2	5.50	1.45	124.1	30.6	161,000	1.4	45.6	14	10 mg.
8	26	...	3.19	0.96	151.5	27.7	Cellular*	5.6	18.6	26	20 mg.
9	20	8	3.16	0.72	125.0	35.1	18	10 mg.
10	34	8	4.49.	1.15	130.4	29.9	7	30 mg.
11	16	1	5.5	1.32	109.8	38.0	21	10 mg.
12	38	8	3.77	1.29	116.2	36.7	7	3 c.cm.
13	...	2	4.26	1.01	138.6	27.1	Cellular*	13.4	36.6	8	4 c.cm.
14	30	7	3.02	0.86	127.0	34.9	Cellular*	0.4	49.4	12	30 mg.
15	25	2	7.01	2.12	110.8	29.8	20	20 mg.
16	19	2	3.19	0.77	145.5	28.5	29	10 mg.
17	25	4	3.50	0.85	152.2	27.5	225,000	0.0	32.0	16	30 mg.
18	25	4	4.06	1.04	153.8	25.3	8	18 gr.
19	19	2	2.46	1.14	92.1	23.2	30	10 mg.
20	25	4	6.13	1.89	116.4	23.7	292,000	0.0	48.6	21	12 gr.
21	25	4	6.13	1.89	116.4	23.7	292,000	0.0	48.6	12	18 gr.

* About 100,000 per c.mm.

TABLE II—*concl'd.*

Number.	Age.	Para.	AFTER TREATMENT.				Interval between two counts (days).	RETICULOCTE PEAK.		RATE OF INCREASE OF RED CELLS FOR FIRST TWO WEEKS.	
			HEMOGRAM.					Actual.	Expected.*	Actual.	Expected.†
			Hb. in g.	R.b.c. in mills.	M.c.v. in cu. μ .	M.c.h.c. per cent.					
1	28	1	11.4	3.96	84.5	34.0	33	37.6	38.0	0.798	0.742
2	22	1	11.68	3.96	85.8	31.0	34	31.0	43.0	1.162	0.776
3	20	2	13.20	4.82	87.1	31.7	47	13.8	34.0	0.413	0.711
4	22	3	9.62	2.91	104.8	31.5	27	9.0	22.0	0.630	0.605
5	18	1	11.55	3.76	95.7	31.9	28	11.0	20.0	0.672	0.579
6	20	2	8.41	2.43	115.2	30.3	30	24.0	42.0	0.623	0.769
7	22	2	10.00	2.70	106.6	34.6	12	17.6	23.0	0.728	0.619
8	26	...	7.83	2.43	107.1	30.1	25	14.6	35.0	0.294	0.724
9	20	8	4.49	1.15	130.4	29.9	20	18.6	43.0	0.147	0.776
10	34	8	6.09	1.76	113.6	30.4	8	0.532	0.684
11	16	1	3.77	1.01	138.6	27.1	24	12.0	26.0	0.210	0.647
12	38	8	8.41	2.41	109.0	31.7	10	3.6	25.0
13	...	2	11.41	4.38	82.2	31.7	14	6.0	34.0	0.641	0.713
14	30	7	7.01	2.12	110.8	29.8	35	25.0	34.0	0.966	0.713
15	25	2	10.3	3.10	100.0	33.3	10	25.1	38.0	0.882	0.746
16	19	2	4.06	1.04	114.5	30.1	21
17	25	4	6.23	1.66	120.4	25.3	29	21.0	42.0	0.553	0.765
			5.80	2.27	96.0	31.1	13	17.8	40.0	0.133	0.748
			7.50	3.11	88.1	26.6	6
						27.6	30	13.0	30.0	0.147	0.713
							13	0.655	0.525

* According to Riddle in pernicious anaemia.

† According to Della Vida and Dyke.

CORRELATION BETWEEN DIFFERENT FACTORS.

TABLE III.
Cases treated during pregnancy—group A.

Dose per day :—		30 mg.		20 mg.		10 mg.	
Marrow	...	Megaloblastic.	Unknown.	Megaloblastic.	Slightly megaloblastic.	Unknown.	Megaloblastic.
Case number	...	2	1 16	3 12	4 5 6 11	7 13	8 9 15
Reticulocyte response	...	E	F SL	M E	G M E F.	G F	G F M
of increase of red cells per day for the first week	...	72.	92 20	Over 100	92 60 66 62	71 43	86 63 ...
...	...	G	G D	P	G G F G	G F	F G M M

FOLIC ACID IN NUTRITIONAL MACROCYTIC ANÆMIA.

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INTRODUCTION.

IN 1931, Wills demonstrated the curative effect of autolysed yeast and crude liver extracts in the treatment of *nutritional macrocytic anæmia* (N.M.A.) in India (Wills, 1931). As a result of experimental and clinical investigations, she came to the conclusion that the condition was a food-deficiency disease, aggravated during pregnancy and caused by lack of Castle's extrinsic factor (Wills, 1934). With the purification of liver extracts, however, it was soon realized that refined liver preparations, known to be highly effective in pernicious anæmia, were inactive in patients suffering from N.M.A. and in the analogous experimental monkey anæmia (Wills and Evans, 1938; Wills, Clutterbuck and Evans, 1937). Therapeutic trials showed that the missing factor was none of the known vitamins of the B-group—thiamin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid and para-aminobenzoic acid (Wills, 1945; Moore *et al.*, 1944). Recently, Watson and Castle (1946) studied a group of cases of N.M.A. that did not respond to purified liver extracts but were improved by oral administration of crude liver preparations. They admitted that such cases were not due to extrinsic factor deficiency (Strauss and Castle, 1932) but were presumably the result of deficient intake of another factor—'Wills' factor', as they called it—present in Marmite and crude liver extracts. Finally, Wills (1948) in a discussion of the essential individuality

of N.M.A., sprue and pernicious anæmia suggests that this factor may be folic acid.

While there is no unanimity of opinion as to the effectiveness of highly refined liver extracts in the treatment of N.M.A. (Wills and Evans, *loc. cit.*; Napier *et al.*, 1938; Foy and Kondi, 1939; Trowell, 1943; Sundaram, 1944; Patel and Bhende, 1949), the response to synthetic folic acid appears to be uniformly satisfactory. Spies *et al.* (1948) have thoroughly established the value of folic acid in the treatment of N.M.A. occurring in the Southern States of the U.S.A. and the West Indies, but more information from other parts of the world where deficiency diseases are endemic is urgently needed (Leading Article, *Lancet*, 1948). Das Gupta and Chatterjea (1946), Kemp (1947) and Goodall *et al.* (1948) have all reported good results with folic acid in a total of 21 cases from India. From South India, however, where the diets are known to be gravely deficient (Aykroyd and Krishnan, 1937) and the incidence of anæmia is high, there is but one report of a similar experience (Sundaram, 1948). In the 14 cases studied by this author were also included other macrocytic anæmias and in many of them folic acid and liver extract were administered simultaneously. Out of 7 cases treated with folic acid alone, two showed no response—a remarkably high incidence—but unfortunately the reactive state of the bone-marrow in these refractory cases is not mentioned. In the present communication, it is proposed to submit the results of treatment with synthetic folic acid of six cases of N.M.A. and one case of sprue associated with macrocytic anæmia and to compare them with the results obtained in one case of N.M.A. treated with a potent crude liver extract.* This liver preparation was reported to have yielded good results in a large number of cases of N.M.A. among Indian prisoners-of-war (Walters, Rossitter and Lehmann, 1947).

CLINICAL MATERIAL.

Out of 45 cases of anæmia admitted to the Nutrition Clinic of the Laboratories during the year 1947-48, 7 patients with uncomplicated typical N.M.A. and 1 patient with macrocytic anæmia of sprue were selected for this study. The nutritional cases belonged to the local plantation-labour class among whom a survey conducted concurrently (Ramalingaswami and Patwardhan, 1949) revealed, among other deficiencies, sub-optimal intakes of B vitamins and animal protein products. The ages of the patients ranged from 22 to 55 years, and 3 of them were women. They were found to be free from malarial and helminthic infections, showed presence of free hydrochloric acid in their gastric contents, and none exhibited signs of spinal-cord involvement. The peripheral blood picture was distinctly macrocytic in every case and the bone-marrow showed a 'megaloblastic arrest' (but see below under Discussion). They all complained of lack of appetite, weakness and fatigue. Diarrhoea was a prominent symptom and was present in 7 cases, 3 of them suffered from mild peripheral neuritis, 2 had oro-lingual signs of riboflavin deficiency and 1 had Bitot's spots. Pitting oedema of the lower limbs was present in 6 patients. With the exception of the sprue patient, none had been treated in the past for their current illness.

* T.C.F. crude liver extract.

METHODS AND DOSAGE.

All patients were admitted to hospital. A detailed dietary and medical history was obtained and a careful physical examination was conducted in each case. The red and white cell counts and determinations of hæmoglobin [by Wong's (1928) method using a photo-electric colorimeter] and of the packed cell volume (using Wintrobe's hæmatocrit tubes) were made on 5 c.c. of venous blood obtained from the antecubital fossa and mixed in a tube containing a suitable quantity of oxalate mixture (Wintrobe and Landsberg, 1935). These observations were recorded in every case initially and weekly after starting treatment, blood being drawn in the recumbent position. Blood smears and reticulocyte counts were made on capillary blood obtained by fingerprick. The reticulocytes were counted against red cells by using 0.3 per cent brilliant cresyl blue in absolute alcohol. The counts were performed every day until the peak was reached. Films of bone-marrow obtained by sternal puncture prior to treatment were stained with May-Grunwald-Giemsa (Cowdry, 1943) and differential counts performed on 500 nucleated cells (results given in Table IV). Analyses of the stomach contents for free and total acid were made on (a) the fasting secretion, (b) after feeding 100 c.c. of 7 per cent ethyl alcohol and (c) after subcutaneous administration of 0.5 mg. of histamine acid phosphate.

Throughout the period of observation the diet was strictly controlled and consisted of the following daily menu: Two rice meals with vegetable soup, 4 slices of bread and butter, weak tea and an orange. Synthetic folic acid (Folvite) was administered in daily oral doses, starting with 20 mg. and then reducing to 10 mg. after the 14th to 21st days. The patients were observed for one to three months on this regimen. No case could be followed for more than 3 months.

RESULTS.

N.M.A. treated with folic acid.—In each of the 6 cases, the effect on the clinical condition was striking. Diarrhoea was controlled within 48 hours, appetite returned between the 3rd and 5th days, and a sense of well-being was manifest from about the 7th day of treatment. Oedema had cleared up completely by about the 20th day and by this time the patients expressed an anxiety to return to work. Burning of the tongue and along the oesophagus, of which 3 patients had complained, were relieved; but angular stomatitis and cheilosis persisted. During the period of observation which ranged from 30 to 80 days, no deterioration in peripheral neuritis initially present in 3 cases was observed. Bitot's spots remained unaffected. The reticulocytes began to rise from the second day, reaching a maximum of 9 to 18 per cent on the 6th to 11th days. This was followed by a steady rise in the red cell count and hæmoglobin, and by a fall, though more gradual, of the mean corpuscular volume to within normal limits. The initial red cell count, which ranged from 1.19 to 2.06 million/c.mm. and averaged 1.67, ranged from 3.36 to 4.36 and averaged 3.93 after 4 weeks' treatment. During this period, the mean hæmoglobin value increased from 7.4 g. to 11.9 g. per 100 c.c. The rise was less sustained thereafter in three cases followed for over one month, reaching a red cell count of 4.39 million and 14.86 g. of hæmoglobin at the end of 50 days in case 2 and 4.86 million and 14.4 g. at the end of 70 days in case 1. A sharp rise in the leucocyte count was evident in those cases associated with an initial value

of less than 4,000 cells/c.mm. The hæmatological data before and during treatment are presented in Table I and one case of the series is described in detail below :—

TABLE I.

Response to folic acid in 6 patients with N.M.A.

Case.	Age.	Sex.	Day.	R.b.c. mill./ c.mm.	Hb. g. per cent.	M.c.v. cu.μ.	W.b.c.	RETIC. PER CENT.		FOLIC ACID.	
								Peak.	Day.	Daily dose, mg.	Days.
1	55	M	1	1.50	6.9	143.0	2,250	18	6	20 10	1-21 22-70
			14	2.78	9.1	115.0	6,800				
			28	3.36	12.7	131.0	5,800				
			42	3.60	13.2	117.5	8,750				
			56	4.72	13.9	89.0	4,850				
			70	4.86	14.4	82.5	6,300				
2	22	F	1	2.06	5.8	116.5	3,200	9	6	20 10	1-14 15-49
			14	3.29	7.2	100.5	10,500				
			28	4.20	8.2	..	8,500				
			42	4.35	11.2	89.5	6,000				
			50	4.39	14.8	96.0	6,350				
3	35	M	1	1.19	6.4	147.0	1,800	10	9	20 10	1-19 20-42
			14	2.65	10.1	109.5	4,000				
			42	3.55	11.8	84.5	5,200				
4	36	M	1	1.46	6.5	123.0	6,200	11	11	20 10	1-21 22-30
			14	3.30	11.2	103.0	3,600				
			28	4.36	13.4	88.0	5,400				
5	30	F	1	1.83	7.8	115.0	5,200	13	10	20 10	1-21 22-30
			14	3.10	10.5	106.5	4,200				
			29	3.89	11.6	95.0	4,400				
6	45	M	1	1.98	10.8	166.6	8,000	18	8	20 10	1-18 19-30
			14	3.00	12.4	110.0	..				
			28	3.84	13.4	98.5	..				

V. Ramalingaswami and P. S. Menon.

N.M.A. treated with folic acid.—Case 1, L. G., a man aged 55 years, was admitted to the Clinic on 18th June, 1948 with a history of easy fatigue and weakness of two years' duration, very insidious in onset. He had been employed as a gardener for 30 years but found himself unable to work within recent months. During the past month he had been suffering from diarrhoea of watery type, followed by numbness and a pricking sensation in the hands and feet and burning of the tongue. His diet for several years had consisted of polished rice, sweet potatoes and occasionally vegetables. Meat was consumed once in a fortnight but he could not afford milk and eggs. There was no history of malaria. Physical examination revealed a well-developed person with marked pallor of the face and neck, red tongue with atrophic papillae, fissured nails, grey hair, hyperpigmentation of the skin and visible mucous membranes, and tenderness of the calf muscles. The reflexes and sensations were unchanged. Liver and spleen were not palpable. He had not been treated before. His blood results were: R.b.c. 1·50 million/c.mm., Hb. 6·9 g., w.b.c. 2,250/c.mm., p.c.v. 21·5 c.c. per cent, m.c.v. 143·3 cu.μ, m.c.h. 46 γγ, m.c.h.c. 31·0 per cent, reticulocytes 1·2 per cent. The sternal bone-marrow showed a typical megaloblastic reaction. Free HCl was present in the resting gastric sample. He was given 20 mg. daily of synthetic folic acid by mouth for 3 weeks and thereafter 10 mg. a day. Diarrhoea was controlled within 2 days, weakness and anorexia gradually lessened, burning of the tongue disappeared and the patient was completely asymptomatic at the end of 3 weeks, although paraesthesia of the hands and feet still persisted but were not aggravated. As can be seen from Table I, a coincident prompt hematological improvement ensued. Reticulocyte crisis occurred at 18 per cent level on the sixth day. In 14 days there was an increase of 1,280,000 red cells and 2·2 g. of haemoglobin; at the end of 63 days the red cell count was 4·84 million and Hb. 14·4 g. No further rise occurred subsequently. The leucocytes increased to within normal limits during the first seven-day period.

N.M.A. treated with crude liver extract.—Case 7, a man aged 54 years, was first seen at the Clinic on 14th July, 1948 with a two months' history of fatigue, loss of 'digestive power', watery diarrhoea, pain and burning of the tongue, retrosternal burning sensation ('as if there were ulcers all the way down the stomach'), and swelling of the feet. The onset was gradual, the first symptom noticed being loss of breath on slight exertion. Physical examination revealed a well-developed person with pale mucous membranes, dry inelastic skin, pitting oedema of both feet and ankles, dry brittle hair, pale tongue with flattened papillae and fissured convex nails. No abnormality of the nervous system was detected; liver and spleen were not palpable. Alcohol test meal revealed hypochlorhydria. Blood studies showed r.b.c. 960,000/c.mm., Hb. 5 g., w.b.c. 3,350/c.mm., p.c.v. 16 c.c. per cent, m.c.v. 165·5 cu.μ, m.c.h. 52 γγ, m.c.h.c. 31·3 per cent, reticulocytes 2 per cent. The bone-marrow was 'megaloblastic'. Six c.c. of crude liver extract were administered intramuscularly every alternate day for a continuous period of 44 days, a total of 132 c.c. being given. The effect on the clinical condition and blood picture was in every respect similar to the above cases, except that the patient suffered pain from repeated injections. Reticulocyte crisis occurred on the 7th day at 12 per cent level and was followed by a continuous rise in red cell counts and hemoglobin levels. The blood findings during treatment are presented in Table II. It will be seen that at the end of 42 days his red cell count was 3·97 million and Hb. 12 g. These figures are nearly identical with an average red

TABLE II.

Response to crude liver extract in one patient with N.M.A.					
Day of therapy.	R.b.c. mill./c.mm.	Hb. g. per cent.	M.c.v. cu.μ.	W.b.c.	Reticulocytes, per cent.
					2·0
					2·8
					6·2
					12·4
					8·0
					2·4
					1·8
1	0·96	5·0	165·5	3,350	..
3
4
6	..	7·5	..	4,750	..
10	1·21	7,850	..
13	..	9·4	120·0	7,300	..
17	2·13	10·8	93·4	4,850	..
34	3·96	12·0	93·1		
44	3·97				

cell count of 3·83 million and 12 g. Hb. reached at the end of the same period in 3 patients of the group receiving folic acid. The greater increase in red cells and Hb. observed in this patient can be explained by his lower initial figures.

Macrocytic anæmia of sprue treated with folic acid.—In contrast to the low economic status and poor dietary history of the above cases, R. S., Case 8, a well-to-do woman aged 30 years, called at the Clinic on 14th April, 1948 complaining of diarrhœa, sore-mouth, weakness and fatigue of 6 years' duration. During this period, she had had several relapses of diarrhœa with pale bulky foul-smelling stools, without blood or mucus. She had been previously unsuccessfully treated with bismuth, emetine, liver and iron preparations. She had lost considerable weight, her menstrual flow became scanty and the abdomen was distended. On examination, she was a pale, emaciated woman, weighing 72 lb., tongue showed denudation of mucous membrane at the tip and margins, abdomen was distended and tympanitic, there was no evidence of free fluid and liver and spleen were not palpable. Cardio-respiratory and nervous systems showed no abnormality. On a diet containing 40 g. of fat daily for 3 days, total fat in the dried stool was 34·9 per cent. No cellular exudate was present on microscopic examination and no pathogenic organisms were isolated on culture. Her initial red cell count was 2·77 million/c.mm., Hb. 11 g. per cent, w.b.c. 6,850/c.mm., p.c.v. 34 c.c. per cent, m.c.v. 122·7 cu.µ, m.c.h. 39·6 γγ, m.c.h.c. 32·3 per cent, reticulocytes 1·43 per cent. Bone-marrow showed a megaloblastic hyperplasia. Alcohol-fast but not histamine-fast achlorhydria was present. Twenty mg. of folic acid a day were given by mouth for three weeks and the dosage then reduced to 10 mg. By the fourth day her general condition greatly improved, appetite returned, there was no burning of the tongue and the frequency of the stools was reduced to twice a day. The improvement was maintained throughout the five weeks' period of trial at the end of which time her weight had increased by 12 lb. The effect on the blood picture is presented in Table III. It will be noted that at the end of five weeks of folic-acid treatment, there was practically no change in the level of hæmoglobin, although a slight increase in the red cell count occurred, and the mean corpuscular volume returned to within normal limits. The absence of hæmopoietic response, despite dramatic symptomatic relief, in treating the sprue syndrome with folic acid was remarked by Davidson, Girdwood and Innes (1947) and by Morrison and St. Johnston (1947). The patient was then treated with proteolysed liver powder* (3 teaspoonfuls twice daily) for a further period of 1 month. This resulted in a slight rise in the red cell count but the Hb. remained unaffected.

TABLE III.

Response to folic acid in one patient with macrocytic anæmia of sprue.

Day of therapy.	Treatment.	R.b.c. mill./c.mm.	Hb. g. per cent.	M.c.v. cu.µ.	W.b.c.	Reticulocytes, per cent.
1	Folic acid 20 mg./day	2·77	11·0	122·7	6,850	1·43
5	"	3·06	10·1	104·5	6,900	4·00
11	"	3·32	10·1	96·4	6,400	9·5
18	"	3·53	10·1	90·6	6,800	3·2
21	Folic acid 10 mg./day	3·62	11·6	87·0	4,950	..
28	"	3·68	11·6	86·9	7,500	..
35	"	4·03	10·8	81·8	5,600	..
36	Proteolysed liver ..	4·10	10·8	95·1	10,800	..
50	"					
66	"					

DISCUSSION.

The patients in the small series described above had typical uncomplicated N.M.A. and the results clearly show that synthetic folic acid administered orally will bring about a prompt improvement in their clinical and hæmatological status

* T.C.F. Prohepex.

which is in every way comparable to the parenteral administration of a potent crude liver extract. The convenience of medication by mouth makes folic acid the drug of choice, although the hæmopoietic effect was not found to be superior as claimed by Goodall *et al.* (*loc. cit.*). Comparison of the results with those obtained in pernicious anaemia treated with folic acid (Davidson and Girdwood, 1947; Wilkinson, 1948) also shows that the improvement in the blood picture in both conditions is similar. It is of interest that three patients with peripheral neuritis at the beginning of treatment showed no subjective or objective deterioration at the end of the test period. Case 1, who was followed for over 80 days, showed no change in his neurological condition. Davidson and Girdwood (1948) noted that severe multiple neuritis developed in some pernicious anaemia patients treated with folic acid. The neuritis, although recent, showed no response to administration of thiamin hydrochloride, which indicates that the mechanism may not be through an upset in the balance of B vitamins. The maximum reticulocyte counts attained by the patients were in general lower than those observed by others. Macrocytosis of the red cells persisted for 14 to 22 days, a finding similar to that of Das Gupta and Chatterjea (*loc. cit.*). It should be noted that the diets were restricted throughout the period of study and it is likely that higher blood figures and quicker rehabilitation could be achieved by simultaneous institution of diets high in calories and rich in essential nutrients. The dosage of folic acid adopted here appears to be adequate.

The bone-marrow morphology in N.M.A.—In the discussion that follows, the nomenclature is that of Israels (1941) and the term megaloblast is confined to a series of pathological cells with a distinctive nuclear structure as seen typically in Addisonian pernicious anaemia in relapse (Israels, 1939). It is often stated that the bone-marrow in N.M.A. is megaloblastic and indistinguishable from pernicious anaemia and that gastric achlorhydria, in the absence of spinal cord degeneration, is the only point of distinction (Spies, 1947; Spies *et al.*, *loc. cit.*). In the present series, the bone-marrow of the patient with sprue and of four others with N.M.A. showed a typical megaloblastic reaction (Table IV):—

TABLE IV.

Differential counts on nucleated marrow cells (percentage frequency).

Case number:—	1	2	3	4	5	6	7	8
Total white cell series	41	52	50	39	47	58	45	48
Total red cell series	59	48	50	61	53	42	55	52
Hæmocyto blasts and other unclassified blast cells.	3	1·8	1·5	2	2	1	1·4	1·8
Pro-erythroblasts	8	..	5·2	2
Early normoblasts	10	6	31	4	30	25	5	4
Intermediate normoblasts	5	8	4	3	6	8	6	2
Late normoblasts	4	6	2	1	4	3	3	1
Basophilic megaloblasts	16	8	..	16	19	14
Polychromic megaloblasts	16	14	3	28	3	2·4	15·4	19
Orthochromic megaloblasts	4	4	..	7	1	..	4·6	10
Megakaryocytes	1	0·2	0·5	..	1·8	0·6	0·6	0·2

EXPLANATION OF PLATE VI.

- Fig. 1. (*Case No. 1*, bone-marrow, May-Grunwald-Giemsa stain, \times 1,150).
Megaloblasts of Ehrlich's type showing varying grades of cytoplasmic ripening.
- „ 2. (*Case No. 3*, bone-marrow, May-Grunwald-Giemsa stain, \times 1,150).
A group of erythroblasts of the early normoblastic type.
- „ 3. (*Case No. 2*, bone-marrow, May-Grunwald-Giemsa stain, \times 1,150).
A group of basophilic megaloblasts with commencing hæmoglobinization.
- „ 4. (*Case No. 5*, bone-marrow, May-Grunwald-Giemsa stain, \times 1,150).
Early normoblastic reaction.
- „ 5. (*Case No. 7*, bone-marrow, May-Grunwald-Giemsa stain, \times 1,150).
A group of polychromic megaloblasts.
- „ 6. (*Case No. 8*, bone-marrow, May-Grunwald-Giemsa stain, \times 1,150).
A group of orthochromic megaloblasts.

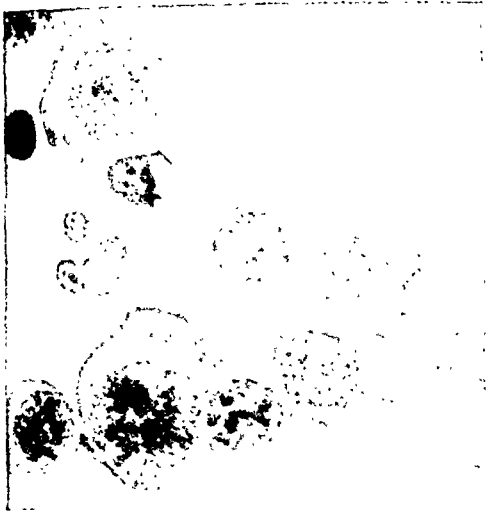


Fig. 1.

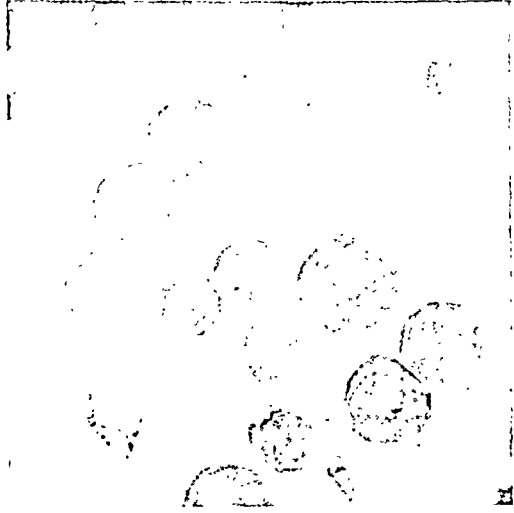


Fig. 2.



Fig. 3.

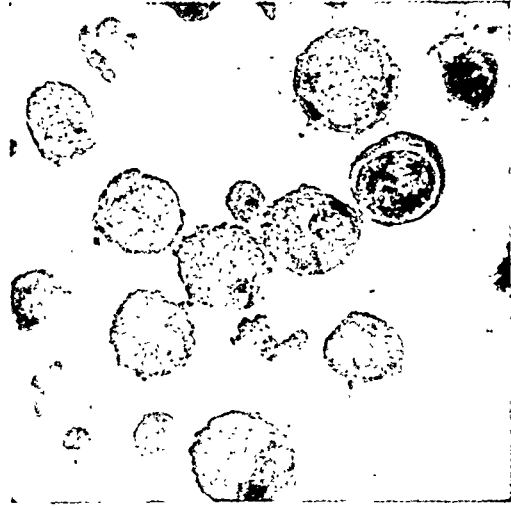


Fig. 4.



Fig. 5.

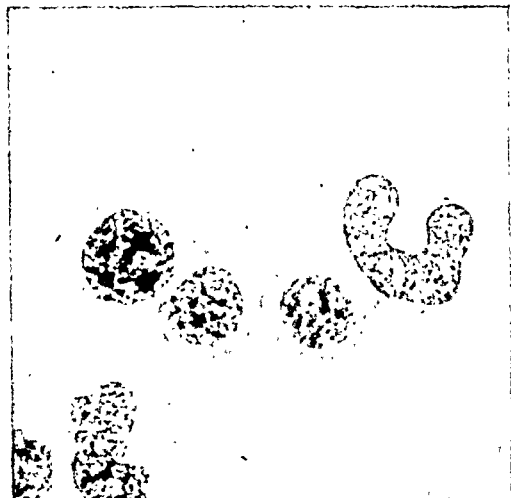


Fig. 6.

SUMMARY AND CONCLUSIONS.

1. Six patients with typical uncomplicated N.M.A. were treated with synthetic folic acid by mouth. The clinical and hæmatological improvements were striking and identical with the response obtained in another patient with N.M.A. treated with crude liver extract by intramuscular injection.

2. Treatment of one patient suffering from sprue in association with macrocytic anæmia resulted in prompt symptomatic relief but was not followed by any appreciable hæmopoietic response. Subsequent treatment with proteolysed liver for one month also produced no change in the hæmoglobin value.

3. The morphological types of erythropoiesis in N.M.A. are discussed in the light of recent advances.

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A PRELIMINARY STUDY OF THE TOXICITY OF *CALOTROPIS GIGANTEA*.

BY

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Calotropis gigantea, belonging to the N.O. *Asclepiadaceæ*, is an erect perennial shrub occurring throughout India, and is commonly known by the following names in the various Indian languages: Arka (Sans.); Madar (Hindi); Akanda (Bengali); Erukku (Tamil); Jelludu Chettu (Telugu).

It grows chiefly in wasteland, requires neither cultivation nor proper attention and withstands dryness for several months. The bark is pale, and young shoots are covered with a white tomentum; leaves are subsessile, oblong, acute or acuminate; flowers are dull-purple or purplish or white, umbel or corymbs; seeds ovate with a silk-white coma.

In the indigenous literature it is a reputed medicinal plant, the root bark being considered to be a valuable remedy in skin diseases, enlargement of abdominal viscera, intestinal worms, cough, ascites and anasarca; the milk juice a drastic purgative and caustic, the flowers a digestive stomachic and tonic; all parts of the plant are said to have valuable alterative properties in small doses and emetic in large doses. In South India the milk juice is a favourite application in painful joints and is used in criminal poisoning for infanticide by giving internally and for producing abortion by local application.

Reports have been received from several veterinary officers of the Madras Presidency, including the Principal, Madras Veterinary College, stating that *Calotropis gigantea* possesses toxic effects on animals, the leaves and roots being employed in criminal cattle poisoning. The Officer in-Charge of the Veterinary Hospital, Poona City has reported that the various parts of the plant are useful as counter-irritant, vermicide, cathartic, expectorant, stimulant and caustic.

No detailed work appears to have been done on this plant.

With a view to investigate the toxicity of the plant and to establish its therapeutic usefulness in veterinary practice research was undertaken under the auspices of the Indian Council of Agricultural Research in the Scheme for Research into Indigenous Drugs of India relating to veterinary practice with special reference to toxicology on the following lines :—

1. Feeding experiments on dogs, cattle and sheep.
2. Isolation of the active principle by chemical analysis.
3. Pharmacological actions of the active principle or principles.
4. Search for antidotes.
5. Therapeutic trials.

Material.—The plant which is growing in abundance around the city of Madras was obtained in large quantities, dried in shade, leaves and flowers separated and powdered apart and stored. The milk juice, used for isolation of the active principle, was obtained fresh whenever needed, by collecting the various drops that exudes when the leaves are plucked.

Feeding experiments (leaf powder).

Thirteen dogs were fed with the powder of the dried leaves in varying doses ranging from 1/8 g. to 4 g. per kilo body-weight. The train of symptoms produced was all similar except for the severity according to the dose. Fifteen minutes after administration the dog becomes uneasy, burrowing the ground with the fore paws. Salivation begins, which becomes excessive, foaming at the mouth. This was followed in 5 minutes by severe retching and vomiting of the whole drug given, leading to prostration of the animal. On an average the interval between the time of administration and the act of vomiting was found to be 30 minutes. The drug was usually given suspended in water by stomach tube. Administration of the leaf powder in warm water hastened the train of symptoms and vomiting was produced in 25 minutes. Hypodermic injection of atropine, administration of demulcents as gum acacia and treacle before the administration of the powder delayed emesis considerably, by 30 minutes or more.

Dose of $\frac{1}{4}$ g. and $\frac{1}{2}$ g. per kilo body-weight, which were toxic to dogs, did not exhibit any symptom in calves. Similarly, doses of $\frac{1}{2}$ g. and 1 g. of the leaf powder per kilo body-weight did not reveal any change in the normal behaviour of goats (Table I).

Aqueous extract of the leaf powder.—The aqueous extracts were prepared by boiling the powdered leaves with water and decanting the fluid successively 3 or 4 times. The extract obtained by decantation was concentrated to 3 to 4 ounces by evaporating the water content.

A single dog fed with the aqueous extract equivalent to one gramme of powder per kilo body-weight produced the same symptoms as the leaf powder.

With the residue, however, which was given to another dog, slight restlessness which passed off in 5 minutes was the only symptom noticed.

Cumulative action.—To determine the cumulative action of the drug, several dogs, 2 calves and 2 goats were tried with daily administration of the powdered leaf in varying doses (Table II).

TABLE I.

Feeding experiment to find out the toxicity of Calotropis gigantea on canines and ruminants.

Number.	Animal.	Weight, kilos.	Dose, g. per kilo.	Quantity, g.	Nature of the material.	Symptoms.
1	Dog	6.2	1/8	0.75	Leaf powder	Restless after 15 minutes, scratching and burrowing into the sand. Salivation starts immediately which increases resulting in retching and emesis in 30 minutes after the administration.
2	"	4.4	1/8	0.55	"	
3	"	5.2	$\frac{1}{4}$	1.25	"	
4	"	6.8	$\frac{1}{2}$	3.4	"	
5	"	4.7	1	4.7	"	
6	"	4.0	1	4.0	"	
7	"	5.2	1	5.0	"	
8	"	4.6	2	9.0	"	
9	"	4.5	2	9.0	"	
10	"	6.7	3	20.0	"	
11	"	7.5	4	30.0	"	
12	"	7.4	$\frac{1}{4}$	1.9	" powder in warm water.	
13	"	7.5	$\frac{1}{2}$	3.8	Leaf powder in warm water.	Emesis produced in 20 minutes.
14	"	6.5	1	6.6	Aqueous extract	Vomited in 30 minutes. No emesis. No symptoms.
15	"	7.2	1	6.6	Residue	
16	Calf	55 approx.	$\frac{1}{4}$	14	Leaf powder	
17	"	55	$\frac{1}{4}$	28	"	
18	Goat	20	$\frac{1}{4}$	10	"	
19	"	20	1	20	"	

TABLE II.

Feeding experiment on canines and ruminants to determine the cumulative toxicity of Calotropis gigantea.

Number.	Animal.	Weight, kilos.	Dose, g. per kilo.	Quantity, g.	Nature of the material.	Number of days administered.	Alive or dead.	Observations.
1	Dog	6.2	1/8	0.75	Leaf powder	13	Died	<p>The symptoms accompanying the single dose, namely restlessness, salivation, retching and emesis, were more violent on the succeeding days. Lack of appetite, quick deterioration in the condition and loss in weight caused death in a few days. Post-mortem findings were as follows:—</p> <p><i>Stomach and intestines</i>: severe congestion and superficial necrosis of the mucous membranes of the stomach, small and large intestines.</p> <p><i>Spleen</i>: congested.</p> <p><i>Liver</i>: enlarged, congested and friable. Small areas of necrosis observed in certain cases.</p> <p><i>Kidneys</i>: congestion of the vessels and necrosis of the mucous membranes of the tubules.</p>
2	"	4.4	1/8	0.55	"	10	"	
3	"	5.2	1/4	1.25	"	7	"	
4	"	6.8	1/4	3.4	"	6	"	
5	"	4.0	1	4.0	"	5	"	
6	"	4.5	2	9.0	"	4	"	
7	"	6.7	3	21.9	"	2	"	
8	"	7.5	4	30.0	"	3	"	
9	"	6.5	1	6.6	Aqueous extract	5	"	
10	"	7.2	1	6.6	Residue left after extraction.	5	"	
11	Calf	55	1/4	14	Leaf powder	34	Alive	<p>Gradual improvement in general health with increased appetite, glossy skin and increase in weight.</p>
12	"	55	1/4	28	"	23	"	
13	Goat	20	1/4	10	"	25	"	
14	"	20	1	20	"	22	"	

Dogs which were given daily doses of 1/8 g. to 4 g. per kilo body-weight lost in condition and body-weight quickly, the appetite was deprived, dullness and prostration supervened resulting in death from 3 to 7 days. The symptoms produced by a single daily dose were quickened and intensified on the succeeding days. On post mortem severe congestion or superficial necrotic changes of the mucous membranes of the stomach, intestines and rectum were noted. The liver was enlarged and congested, friable and small areas of necrosis were occasionally found. The spleen was congested. Congestion of the cortical and medullary vessels of the kidneys, and degenerative changes of the tubules were also common.

Nothing unusual was found with lungs and heart.

The aqueous extract of the residue produced the same results as the leaf powder.

As against the canines, ruminants as calves and goats, which were fed with 1/4 g. to 1 g. of the powder per kilo for more than 20 days to a month, did not reveal any harmful effect. On the other hand an increased appetite, glossy skin and better healthy condition of the animals observed were noteworthy.

From the above feeding experiments it is clear that there is a certain irritant principle in the leaves partially soluble in water, which by irritation of the gastric mucosa produces reflex salivation and emesis. Delayed emesis caused by prior administration of atropine and mucilages may be attributed to the anti-sialagogue action of the former and the demulcent action of the latter which antagonizes the irritant action of the leaf powder on the gastric mucosa. The active principle has been found to be cumulative, thereby damaging the liver and the tubules of the kidney during accumulation and excretion, and death being attributed to the cumulative toxic action of the same.

The failure to produce the above harmful effects in the case of ruminants is probably due to the complexity of the stomach and large volume of the rumen, where most of the drug might get itself either de-toxicated or distributed in a large area whereby concentrated action is impossible. Its tonic action on the general health of the animal is to be investigated by subjecting the active principle to pharmacological actions of the various organs.

Isolation of the active principle by chemical analysis.

Chemical work was undertaken in order to isolate the poisonous principle of the plant from the latex and also from the leaves of the plant. Working on the leaves it was possible to obtain a fraction which had the same pharmacological reactions as that of the active crystalline principle isolated from the milky juice.

The procedure for the investigation on the leaf powder was as follows :—

Material for this investigation consisted of leaves obtained from the local area dried in the shade and powdered.

Preliminary examination.—Fifty grammes of the powder were extracted with water and the aqueous extract gave the following reactions :—

1. It was faintly acidic to litmus.
2. It gave no reaction with FeCl_3 indicating absence of tannins.

3. It gave faint-reducing reaction with Fehling's solution.

4. The acidic aqueous extract gave no precipitate with Mayer's and other alkaloidal reagents.

Extraction with different solvents.—In order to have a general idea of the nature of the constituents contained in the powder, 20 g. of the ground material were extracted in a Soxhlet's apparatus, successively with various solvents when the following amounts of extracts dried at 100°C. were obtained :—

	Per cent.
Petroleum ether	1.28
Ethyl ether	0.82
Chloroform	1.98
Ethyl acetate	0.54
Ethyl alcohol	16.82

Detailed investigation.—One kg. of the leaf powder was extracted with cold alcohol by percolation to exhaust. The alcohol was distilled off under reduced pressure and the residue taken with water. The volume of the aqueous liquid was 300 c.c. A dark resinous solid was separated. The clear aqueous liquid was then extracted with : (i) petroleum ether, (ii) ether, (iii) chloroform and (iv) ethyl acetate.

(i) *Petroleum ether residue.*—The residue was refluxed with alcohol and cooled. The insoluble fatty matter separated as an oily layer which was collected in a dish. The insoluble fraction consisted of fats and oils.

(ii) *Ether residue.*—The residue got by extracting with ether was resinous brownish matter and was dark in colour and was thrown down on adding water. The resin was soluble in alcohol.

(iii) *Chloroform extract.*—The residue from the chloroform extract was dissolved in alcohol and diluted with water. Resinous substance was separated. The solution was turbid even after filtration. It was then extracted with ethyl acetate. This fraction was kept in a dish, but nothing definite was separated.

(iv) The aqueous solution was extracted with ethyl acetate, but nothing definite was separated.

Aqueous liquid.—The aqueous extract was then worked up as in lead method. The aqueous liquid was treated with lead acetate solution. The lead precipitate was separated by filtration and collected. The filtrate was freed from lead by passing hydrogen sulphide in the solution. The solution which was then filtered and freed from lead sulphide was concentrated on a water-bath to a syrupy consistency and kept in a desiccator. Nothing definite was separated from this.

The lead filtrate was bitter to taste. It was divided into two parts according to the solubility in alcohol. The fraction which was soluble in alcohol was freed from alcohol and again extracted with ethyl acetate. The ethyl-acetate extract was then concentrated by removing ethyl acetate by distillation and the residue dissolved in a little amount of water and filtered. The filtrate was then tried for pharmacological experiments and it showed similar actions as those of the active principle of the milky juice.

The lead precipitate was taken and was well refluxed with alcohol. The alcoholic solution was freed from lead by passing hydrogen sulphide. The solution was thus freed from lead and evaporated *in vacuo* in a desiccator over concentrated sulphuric acid.

The concentrate was then tested. It was acidic to litmus. It gave reducing reaction with Fehling's solution, but it never gave reducing reaction after hydrolysis.

Madar juice.—As the juice of *Calotropis* is very poisonous and as according to Dymock many accounts have been given of various preparations from the juice, attempt was made to isolate the crystalline principle. The method adopted was as follows :—

Madar juice is a white milky liquid and has specific gravity of 1.021 to 1.024 and the total solids amount to about 14.8 per cent. On heating it separates into a whitish clot and a straw-coloured serum.

Isolation of poisonous principle.—Madar juice was heated on a boiling water-bath to effect coagulation. The clear yellowish serum which separates was filtered; the clot washed with boiling water and the washings added to the serum.

The serum was then worked with solvents like petroleum ether and ether. The extracts obtained in ether and petroleum were resinous rubber-like substances.

The serum was then repeatedly extracted with ethyl acetate, the extract being collected in another separating funnel. The total ethyl-acetate solution was then washed with small quantities of distilled water. The ethyl acetate was then distilled off over a boiling water-bath and the residue taken up with hot ethyl alcohol. The ethyl-alcohol solution was concentrated and set aside for crystallization. Crystals of brownish colour were obtained along with the brownish resinous substances. The brownish matter was washed and removed by dissolving in acetone. The crystals were then collected and re-crystallized. White crystals were obtained after two re-crystallizations. The melting point of these crystals was found to be 238°C.

Pharmacological actions of the active principle.

Materials.—

- (i) An amorphous, water-soluble substance, obtained from the leaves by the method described above, was made into solutions of desired strengths, kept in the frigidaire and utilized when required.
- (ii) The crystalline substance from the milky juice of the plant was first dissolved in a few c.c. of 95 per cent alcohol and further dilutions effected in distilled water. The solutions were stored in the frigidaire and taken out when required.

Action of the amorphous substance of the leaves.

On circulation.—2.5 mg. of the substance ($\frac{1}{2}$ c.c. of a 0.5 per cent solution) injected into the femoral vein of dogs, weighing about 3.5 kg. under paraldehyde

anæsthesia, produced an immediate rise of the carotid blood-pressure which was sustained for 15 minutes. The rise was steep but the fall was very gradual (see Plate VII, Graph 1).

The rise is due in part to peripheral stimulation of the heart and in part to the peripheral vasoconstriction. The share of the latter is shown by the observations that the volume of splanchnic organs, such as spleen, decreased as the blood-pressure rose. Similar actions were reproduced even in dogs which have been decerebrated, thereby eliminating central stimulation.

On mammalian heart.—Dogs under paraldehyde anæsthesia were decerebrated under artificial respiration. A portion of the sternum and ribs were cut and removed. Pericardium was slit open and made into a cradle, for the heart, by stitching it to the sides. The right auricle and the apex of the heart were hooked with small curved pins and attached by means of strings to heart levers writing on the moving kymograph drum. When the drug was injected into the femoral vein in the same dose mentioned above, an immediate stimulation of the heart was observed. Increased frequency and amplitude were marked. Similar actions were produced in the cat also (see Plate VII, Graph 2).

Half an hour later 0.13 mg. of atropine was injected into the femoral vein. Repetition of the drug produced the same results.

Isolated frog's heart.—Perfusion of isolated frog's heart with Ringer solution containing the drug in a concentration of 1 in 10,000 slowed the rate but increased the force of ventricular contractions as shown by the increased excursions. The passage of impulses from the auricle to ventricle appears to be interfered with, as evidenced by the partial heart block noticed.

Actions of the crystalline principle obtained from the milky juice.

The pharmacological actions of this principle resemble in nature that of the former but are more powerful.

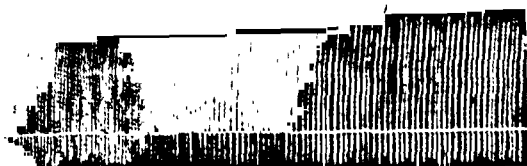
On circulation.—0.25 mg. of the substance (0.1 c.c. of a 0.25 per cent solution) has been found to produce a similar rise in carotid blood-pressure which was sustained for 20 to 25 minutes and the rise was due to the peripheral stimulation of the heart and peripheral vasoconstriction (see Plate VIII, Graph 3).

That the peripheral action on the heart and blood vessels was on the musculature was shown as follows :—

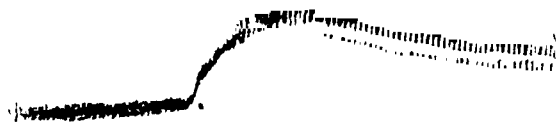
The sympathetic vasoconstrictors of a dog kept under artificial respiration were paralysed by slow injection of 15 mg. of ergotoxine (@ 5 mg. per kilo body-weight) into the femoral vein (confirmed by a dose of adrenaline which produced a fall in the blood-pressure). A dose of 0.25 mg. of the active principle was then given which continued to cause the same rise in blood-pressure as before (see Plate VIII, Graph 4).

On the blood vessels.—One of the hind legs of an anæsthetized dog was perfused with warm oxygenated normal saline solution. The perfusion was done through a cannula introduced into the femoral artery and the outflow was taken out through the femoral vein and allowed to drop on an Inchley's drop recorder which was

PLATE VII.



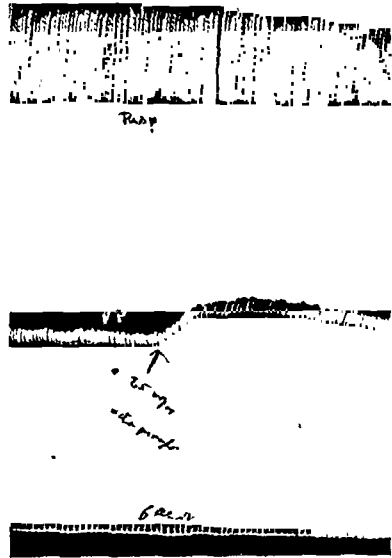
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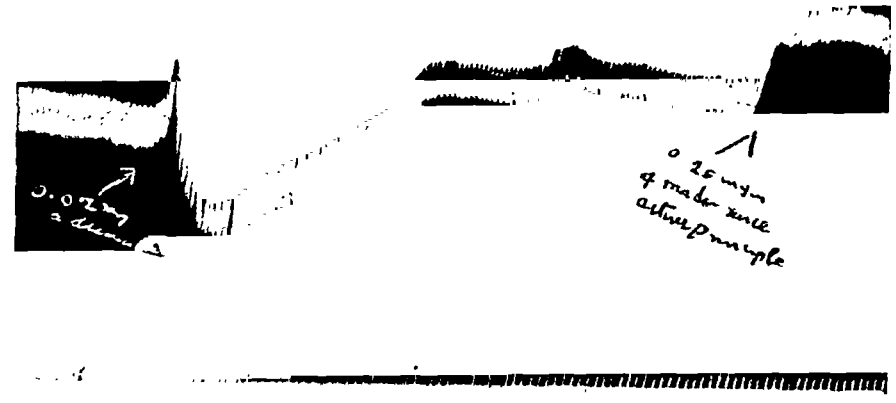
GRAPH 2.—A record of the myocardiogram of a decerebrated cat under chloralose anaesthesia. Note the stimulation of the auricle and the ventricle and the rise in carotid blood-pressure after an injection of 1 mg. of the active principle of the leaves. (Down strokes are contractions. Time 6 seconds.)

GRAPH 1.—A record of the carotid blood-pressure and respiration of a bitch 3·8 kg. body-weight under paraldehyde anaesthesia. Note the rise in the blood-pressure after an injection of 2·5 mg. of the amorphous active principle of the leaves. (Time 6 seconds.)

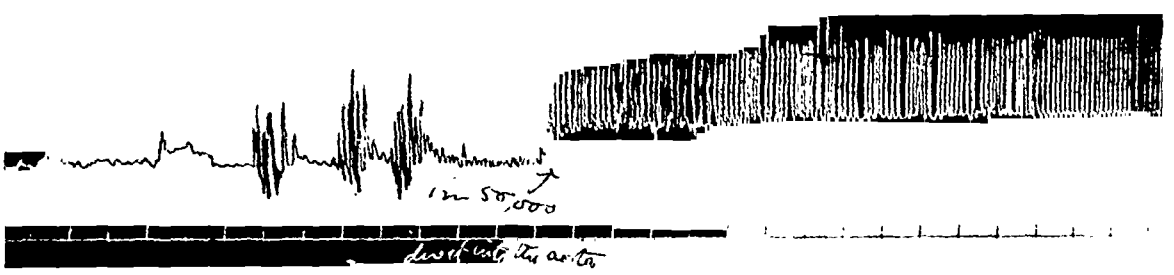
PLATE VIII.



GRAPH 3.—A record of the carotid blood-pressure, respiration and spleen volume of a dog of 2.4 kg. body-weight under paraldehyde anaesthesia. Note the persistent rise in the blood-pressure and the corresponding decrease in the spleen volume after an injection of 0.25 mg. of the crystalline principle of the Madar juice.



GRAPH 4.—A record of the carotid blood-pressure of a dog under paraldehyde anaesthesia after paralysing the sympathetic vasoconstrictors by ergotoxine. Note the rise in blood-pressure after an injection of 0.25 mg. of the crystalline active principle of the milk.



GRAPH 5.—A record of the movements of the ventricle of a perfused kitten's heart. Note the failing heart and the revival of the failing heart after injection of one c.c. of 1 in 50,000 solution of crystalline principle into the aorta. (Time 6 seconds.)

connected by means of a pressure-tubing to a tambour carrying a writing lever adjusted to record on a kymograph moving at a medium speed. Injection of the drug into the arterial cannula caused considerable reduction in the venous outflow. This was indicated by a fall in the frequency of the record of drops on the kymograph. The results suggest that the drug exerts a vasoconstrictor action.

Isolated mammalian heart.—A kitten was stunned by a severe blow on the head, the chest opened immediately, the heart removed and perfused with oxygenated Locke's solution kept at 37.8°C. Concentrations of the substance to the extent of 1 in 400,000 in the Locke's solution caused an immediate increase in the frequency and amplitude of the ventricular beat. The heart continued to work for 45 minutes, except for slight irregularities of missing a beat occasionally. The quantity of the drug used equals 0.25 mg.

Higher concentrations as 1 in 200,000 produced an immediate increase in frequency and force, soon followed by lessening of the frequency maintaining the same force. Occasionally missing of a beat was noticed. The heart returned to normal after 15 minutes. The total quantity of the substance used was 0.05 mg.

Still higher concentrations, 1 in 10,000 in the Locke's solution, produced an immediate stimulation both in frequency and force. The frequency lessened in a few seconds without affecting the force, the heart stopping in 7 minutes. The quantity of drug used was 0.5 mg. This dose is probably toxic.

The above experiments go to prove that the drug has a direct stimulant action on the heart muscle.

The irritability of the cardiac muscle was increased by the crystalline principle. The minimal effective current for stimulating the heart of a frog was found to be considerably decreased as shown below :—

DISTANCE OF SECONDARY FROM THE PRIMARY COIL IN
CENTIMETRES :

Number.	Before the drug.	Dose.	After the drug.
1	10.5	0.0025 mg.	11.5

The effect of the drug on the latent period was studied in frogs. A stannius preparation was made by putting a soft clamp over the white crescent and the ventricle was stimulated by minimal induced shocks. The normal interval between the moment of stimulation and the beginning of contraction was measured. After removing the clamp an injection of the drug was given intrahepatically; the crescent was again clamped, the ventricle stimulated and the interval between stimulation and contraction measured as before. Studied according to this method it was found that the drug decreases the latent period considerably.

The influence of the drug on the refractory period of the heart was studied in the frog. The technique used was that mentioned by Waddell (1924). A

straight wire was attached to the armature of a signal magnet and was so arranged as to pull the writing lever away from the drum as soon as the circuit was made. The magnet was taken in the primary circuit and the surface of the heart was stimulated by platinum electrodes from the secondary coil. Normally, a stimulus applied anywhere in the diastolic phase produces a contraction and compensatory pause, while the heart is refractory throughout the systolic phase. This substance has been found to decrease the refractory period.

Intestinal movements in an intact animal were studied after giving the drug intravenously in anæsthetized animals. It has been found not to possess any marked action on the intestinal musculature.

Kidney volume.—One-tenth of a mg. of the drug did not produce any appreciable change in the kidney volume, but heavier doses like 1 mg. did produce a decrease of the kidney volume corresponding to the rise in blood-pressure.

Diuresis.—The diuretic action was studied by directing the urine from one of the ureters of an anæsthetized dog to fall on an Inchley's drop recorder. A transient increase in diuresis was noticed after giving the drug.

Action on rabbit's uterus.—The crystalline principle produces a violent contraction of the uterus both in the intact animal and in an isolated perfused organ; also before and after paralysing the sympathetic nerve-endings by ergotoxine.

Power to revive a failing heart.—During the various trials made one or two cases of failing heart were met with and they were revived by the administration of these drugs. A dog under paraldehyde anæsthesia was kept ready for the experiment on the table. Immediately after connecting the carotid and venous cannulae the blood-pressure was rapidly approaching to zero. But for the immediate administration of 2.5 mg. of the crystalline principle ($\frac{1}{2}$ c.c. of a 5 per cent solution) which was kept for the usual experiment the dog would have died. To our surprise the blood-pressure rose immediately and remained steady for the rest of the experiments.

On another occasion an isolated kitten's heart attached to the perfusion cannula failed to work satisfactorily. The auricles and the right ventricle alone were beating. Fifteen minutes later 1 c.c. of 1 in 50,000 solution of the crystalline substance (0.05 mg.) was injected directly into the aorta. The heart revived, the left ventricle began to work and the heart was definitely stimulated and worked satisfactorily for 20 minutes (see Plate VIII, Graph 5).

Cumulation.—In most of the experiments it is found that the dog would not withstand a second or third intravenous injection of a dose of 0.08 mg. per kg. body-weight. With the second, irregularities of the heart develop, the heart failing with the third injection.

Fixation of the dose.—Minimal active dose is found to be 0.01 mg. per kg. body-weight intravenously. Repetition of the drug five times in the above doses, at half-hour intervals, did not produce any harmful effect on the anæsthetized animal.

0.3 mg. of the drug per kg. of body-weight given through the femoral vein proved fatal in the majority of the cases.

On living animals.—0.25 mg. of the drug given subcutaneously and intravenously and repeated in 24 hours in dogs weighing about 3 kg. to 3.5 kg. was found to be harmless.

Two mg. in 2 c.c. given subcutaneously to goats and calves of medium size, daily for 6 days, did not produce any serious alteration in the normal condition of the animals. Only in one case slight thickening of the skin and swelling at the site of injection which disappeared later was observed.

Further work.—As the drug has a very sustained stimulant action on the circulation, further work on this is likely to give very encouraging results and this depends on the availability of sufficient material to determine the chemical constitution and stability.

CONCLUSION.

Since the leaf powder is found to produce a beneficial tonic action in case of ruminants, which is further evidenced by the stimulant effect on the heart, it may possibly be made use of as a cardiac and general tonic. The optimum dosage can be determined only after chemical identification of the active principles.

SUMMARY.

1. Feeding and pharmacological experiments to determine the toxicity of *Calotropis gigantea* have been carried out.

2. In feeding experiments the leaf powder is found to be *toxic to canines* and *non-toxic to ruminants* such as calves and goats. On the other hand it *exerts a tonic effect on the ruminants*.

3. Two substances which are identical in their physiological activities have been extracted, i.e. an amorphous substance from the leaves and a crystalline substance from the milk juice, the latter being more powerful.

4. The *active principle* of the leaves and of the milk produces a sustained rise in blood-pressure when given intravenously to anæsthetized dogs and the action seems to be directly on the musculature of the heart and blood vessels.

5. It increases the irritability of the frog's heart and decreases the latent period.

6. The refractory period is shortened after the drug.

7. It has been found to possess the power of contracting the uterus both *in situ* and isolated, before as well as after ergotoxine.

8. The crystalline active *principle* of the milk has been found to revive failing hearts.

The authors' thanks are due to the Indian Council of Agricultural Research under whose auspices this work was carried out, and to the Director of Animal Husbandry, Madras for his keen interest evinced in this work. They are also deeply indebted to Dr. C. Vareed, M.B.B.S., M.Sc., Professor of Physiology, Madras Medical College, Lieut.-Colonel R. Krishnaswamy, M.B.B.S., Civil Assistant

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